NOVEL DIAGNOSTIC BIOMARKERS AND THERAPEUTIC OPTIONS FOR NEUROENDOCRINE TUMORS

Nieuwe diagnostische biomarkers en therapeutische opties voor neuroendocriene tumoren

Nuevos marcadores diagnósticos y opciones terapéuticas en tumores neuroendocrinos

Aura Dulcínea Herrera Martínez

Directores:

Universidad de Córdoba
Prof. dr. Justo P Castaño
Dr. María A. Gálvez

Universidad de Rotterdam:
Prof. dr. Leo J. Hofland
Dr. Richard Feelders

TITULO: *NOVEL DIAGNOSTIC BIOMARKERS AND THERAPEUTIC OPTIONS FOR NEUROENDOCRINE TUMORS*

AUTOR: *Aura Dulcínea Herrera Martínez*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

https://www.uco.es/ucopress/index.php/es/
ucopress@uco.es

TÍTULO DE LA TESIS:

"NOVEL DIAGNOSTIC BIOMARKERS AND THERAPEUTIC OPTIONS FOR NEUROENDOCRINE TUMORS" (nuevos marcadores diagnósticos y opciones terapéuticas en tumores neuroendocrinos)"

DOCTORANDO/A: Aura Dulcinea Herrera Martínez

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda ha realizado su tesis doctoral bajo nuestra supervisión en la Universidad de Córdoba y la supervisión de los Drs. Leo J. Hofland y Richard A. Feelders en la Universidad de Rotterdam bajo el régimen de cotutela durante los años 2014-2018. Su actividad investigadora ha sido de carácter translacional, con combinación de investigación clínica y básica. Ha participado activamente en numerosos cursos, congresos nacionales e internacionales, así como en la redacción de artículos científicos que han sido aceptados en revistas de alto índice de impacto. Ha realizado una rotación en el Erasmus Medical Center de Rotterdam durante 17 meses. Su tesis doctoral es un compendio de publicaciones, específicamente 7 artículos originales y 2 revisiones. Durante su formación académica ha mejorado significativamente su capacidad investigadora y de trabajo en equipo, mostrando iniciativa y capacidad de diseño de proyectos de investigación.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 21 de noviembre de 2018

Firma del/de los director/es

Justo P. Castaño

María A. Gálvez Moreno

NOVEL DIAGNOSTIC BIOMARKERS AND THERAPEUTIC OPTIONS FOR NEUROENDOCRINE TUMORS

Nieuwe diagnostische biomarkers en therapeutische opties voor neuroendocriene tumoren

Nuevos marcadores diagnósticos y opciones terapéuticas en tumores neuroendocrinos

Aura Dulcínea Herrera Martínez

TABLE OF CONTENTS

Chapter 1

General Introduction
Diagnosis and Management of Neuroendocrine Tumors

Partially based on:
Neuroendocrine tumors: diagnostic, predictive and prognostic markers
Aura D. Herrera-Martínez.[1,2], Leo J. Hofland[1], Wouter W. de Herder[1], María A. Gálvez Moreno[2], Justo P. Castaño[2] and Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.

Invited Review: Endocrine Related Cancer. Submitted for publication

Medical Treatment for neuroendocrine tumors: current options and future perspectives
Aura D. Herrera-Martínez[1,2], Johannes Hofland[1], Leo J. Hofland[1], Tessa Brabander[3], Ferry A.L.M Eskens[4], María A. Gálvez Moreno[2], Justo P. Castaño[2], Wouter W. de Herder[1], Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.
[3] Department of Radiology & Nuclear Medicine, Erasmus Medical Center, Rotterdam, the Netherlands
[4] Department of Medical Oncology, Erasmus Medical Center, Rotterdam, the Netherlands

Invited Review: Drugs. Submitted for publication

INTRODUCTION

The diffuse endocrine system is composed of neuroendocrine cells dispersed throughout the whole body [1]. These cells, which may be found in isolation or in small aggregates, can give rise to neuroendocrine tumors (NETs) [1, 2]. NETs represent a heterogeneous group of rare, slow-growing neoplasms [3, 4], and comprise 1-2% of all gastrointestinal and pulmonary malignancies [5, 6].

According to the last National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER), the incidence of NETs has increased substantially (1.09/100,000 persons in 1973 to 6.98/100,000 in 2012) [7]. It is not known whether this is a true increase in NET incidence, the result of increased use of (improved) diagnostic procedures, or a combination of both [4, 8]. According to the SEER database, 27.4% of NETs have distant metastasis at diagnosis and 20% have regional infiltration [7]. Other series describe increased metastasis rates (localized and distant metastasis) when NETs are diagnosed (60–80%) [9]. Five to ten percent of metastasized tumors have an unknown primary tumor [10]. Despite the increase in incidence, survival in NET patients has improved, especially for patients with advanced gastroenteropancreatic- (GEP-) NETs [7].

This group of neoplasms displays a wide range of biological behavior ranging from benign to highly malignant growth [11]. In NETs, the overall 5-year survival rate ranges between 57-65% [12, 13], but depends on several parameters including the localization of the primary tumor (75.0% for jejunoileal, 42.9% for pancreatic NETs), the presence of metastasis (51.7% in presence vs 80% in absence of metastasis at initial diagnosis), tumor size, grade and stage of disease [12, 14].

1. Clinical features

NETs may produce specific clinical syndromes due to overproduction of hormones and bioactive peptides. The incidence of functioning NETs ranges from 0.01-8.4 cases per 100,000 habitants per year, depending on the secreted hormone. Carcinoid syndrome (CS) is the most frequent hormone-related syndrome (2-8.4 new cases/100,000 habitants/year) and is predominantly encountered in patients with metastasized midgut NETs [15]. The syndrome is mediated by several active hormones, especially serotonin, and comprises several symptoms, including flushing (94%), diarrhea (78%) and abdominal pain (51%), which is usually related to mesenteric fibrosis [16, 17]. Additionally, patients may present with carcinoid heart disease (CHD), which consists in the deposition of plaques on the endocardial surfaces of valve leaflets, subvalvular apparatus and cardiac chambers. CHD affects especially the right side of the heart and is observed in about 60% of patients with CS [18].

Pancreatic NETs (PNETs) are able to produce pancreatic peptides which can lead to specific hormone syndromes. Among these, the most common is the endogenous hyperinsulinism-related hypoglycemia caused by insulinomas [19]. The glucagonoma syndrome should be

also mentioned and is characterized by necrolytic migratory erythema (NME), diabetes mellitus and weight loss [20]. Furthermore, multiple peptide ulcers may be related to gastric hypersecretion, specifically the Zollinger-Ellison syndrome in patients with gastrinomas [21]. Watery diarrhea may be related to functioning NETs that release vasoactive intestinal polypeptide (VIP) or calcitonin, while somatostatinomas may be asymptomatic or present with diabetes mellitus, cholelithiasis, weight loss, steatorrhea and diarrhea [89-91]. In addition, ectopic hormone production (EHP) may also be observed in NETs. In EHP, adrenocorticotropic hormone (ACTH) producing tumors are most commonly observed, but ectopic release of peptides including corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), antidiuretic hormone, parathyroid hormone-related peptide (PTHrP) and gonadotropins by NETs has also been described [96-98].
The proportion of non-functional NETs is larger than functioning tumors. Non-functional NETs may be discovered incidentally during diagnostic procedures, or present with mechanical symptoms (e.g. bowel obstruction, cough, hemoptisis)[22, 23]. Due to their silent clinical presentation, patients with non-functioning NETs generally present late with large primary tumors and advanced disease [24].

2. Diagnosis

NETs may be diagnosed following symptoms-directed evaluation (in case of functioning tumors), due to non-specific symptoms or incidentally during endoscopic/cross-sectional imaging procedures [23]. Diagnosis should be ideally confirmed by histological evaluation, in which immunohistochemical markers are key points, particularly synaptophysin and chromogranin A [25]. Tumor grade is defined using the Ki67 index and the mitotic index according to the World Health Organization (WHO) grading system. For lung NETs, necrosis is also considered [26, 27].

2.1. Currently available markers for NETs

Currently used biochemical markers in NETs are usually hormones or amines secreted by the enterochromaffin cells, which can be influenced by several factors, including co-existent disease(s) and drugs. These biomarkers add to diagnosis, but are insufficient to accurately diagnose, to identify the primary tumor site or to differentiate tumor grading, especially due to limited sensitivity and specificity [28]. Despite this, some of them are considered for the diagnosis and follow-up of NETs according to several clinical guidelines [29-31]. In Table 1, a summary of the sensitivity and specificity of currently used and novel biomarkers in NETs is depicted.

Chromogranin A (CgA): is a protein present in the secretory granules of normal and neoplastic neuroendocrine cell types,which is released with peptide hormones and biogenic amines, and is also the precursor for functional neuroendocrine peptides [50, 51]. Several

Table 1: Sensitivity and specificity of current and novel neuroendocrine biomarkers

Tumor marker	Primary tumor location	Sensitivity	Specificity
Chromogranin A [32-38]	Non-specific	60-83%	72-85%
Urinary5-HIAA [32, 38, 39]	Midgut	35-68%	90-100%
Pancreatic polypeptide [28, 40, 41]	Pancreas, midgut	31–63%	~67%
Neuron-specific enolase [28, 32, 42]	Non-specific	33%	73%
N-terminal brain natriuretic peptide [28, 43]	Midgut (non-specific for CHD)	87%	80%
Progastrin-releasing peptide [44]	Lung	43%	99%
Paraneoplastic Ma antigen 2 [45]	SB-NETs	46-50%	
DCR [46]	SB-NETs	AUC: 0.74	
TFF3 [46]	SB-NETS	AUC: 0.72	
Midkine [46]	SB-NETS	AUC: 0.71	
Multritranscript genes [47-49]	GEP-NETs	75-98%	

Legend: CHD: carcinoid heart disease; SB: small-bowel, AUC area under the curve.

guidelines recommend plasma CgA measurement during diagnosis, treatment and follow up in GEP-NETs. Baseline and serial CgA may predict clinical outcome, prognosis and tumor response [52], and may be indicative for local progression in patients with liver involvement [32]. Additionally, a progressive decrease in CgA levels may be observed in patients with extensive metastatic spread and loss of neuroendocrine differentiation [53]. However, CgA is elevated in only 60–80% of patients with NETs, has a limited sensitivity of 60-83% and also specificity is relatively low, i.e. 72-85% (Table 1) [32-36, 38, 54-56]. Moreover, proton pump inhibitors, atrophic gastritis and impaired kidney function can induce a rise in CgA levels [38, 57]. The combination of CgA with other diagnostic methods, e.g. somatostatin receptor scintigraphy, may increase its sensitivity (93%) and specificity (81%) [58-60]. Importantly, the sensitivity of CgA depends further on the threshold cut-off [37, 38, 53], NET primary location [37, 61, 62], endocrine associated syndrome [9], disease spread, liver metastases [37, 53, 56, 63] and the used assay [64]. Importantly, different analytical properties of the CgA kits give different performances, a fact that must be taken into consideration when comparing results from different clinical studies.

Neuro-specific enolase (NSE): is a soluble cerebral protein which provides information on neural, neuroendocrine and paraneuronal cells [65]. An increase in NSE levels is thought to be related to a high death rate of cells with neuroendocrine differentiation [32]. NSE is probably the most reliable tumor marker in diagnosis, prognosis and follow-up of small cell lung cancer (SCLC) [66]. This marker may be elevated in 38-40% of GEP-NETs patients, in particular in those with high grade tumors [42, 67]. The specificity of NSE is similar to CgA but with lower sensitivity (Table 1) [42, 68, 69]. NSE levels have been directly associated with tumor differentiation, aggressiveness and size [42, 67]. Despite its limited sensitivity,

NSE is inversely correlated to overall survival (OS) in ENETS TNM stage IV [67] and with shorter progression-free survival (PFS), even if CgA levels are normal [70].

N-terminal brain natriuretic peptide (NT-BNP): is a peptide produced by myocardial cells in response to electrolyte and fluid balance; its serum concentration is usually elevated in mid-gut NETs with a sensitivity of 87% and a specificity of 80% [28, 43]. NT-BNP is in particular used for evaluating CHD and it has been reported that a cut-off value of 260 pg/ml has a sensitivity of 92% and specificity of 91% [71]. Interestingly, it has been suggested that patients with elevated NT-BNP levels combined with increased CgA levels have worse OS when compared to CgA alone [28, 72]. Importantly, NT-BNP is not disease specific, thus further studies for evaluating its applicability in the progression of CHD are still required [71].

5-hydroxyindoleacetic acid (5HIAA): Serotonin, produced by (midgut) NETs, is the most prominent hormone associated with diarrhea and flushes in carcinoid syndrome. Its metabolite, 5-hydroxyindoleacetic acid (5HIAA), measured in 24 h urine is used as a diagnostic and follow up marker [73]. Urinary 5HIAA levels are not directly related to the severity of symptoms and large fluctuations within an individual patient have been described [74]. The specificity of 5HIAA is around 90%, but the reported sensitivity is 35-68% in patients with NETs [32, 38, 39]. 5HIAA is mainly used as an indicator of hypersecretory activity in patients with NETs, especially in midgut NETs [32]. Its prognostic value, however, is limited, some studies have related higher urinary 5HIAA levels with mortality [75], but these results were not reproduced by other studies [39, 73]. Its combination with other markers also failed to predict OS, for this reason, 5HIAA determination is only recommended to assess carcinoid syndrome [71].

Pancreatic Peptide (PP): is a non-specific marker in NETs [76]. Around 63% of PNETs and 18-53% of primary gastrointestinal NETs show increased PP levels [41]. Its determination does not seem to increase the diagnostic performance of other markers like CgA, but changes above 50% in PP serum levels seem to correlate with tumor increase on imaging [56].

Despite the above-mentioned limitations, current biomarkers are regularly used in clinical practice and their accuracy increases when combined. Importantly, specific comparisons between markers are difficult since several publications are based on short heterogeneous cohorts and retrospective analysis. Additionally, the differences between assays limit comparisons and solid conclusions.

2.2 Potential novel diagnostic biomarkers

To improve early diagnosis and follow-up of NETs, several new prognostic and treatment-related biomarkers have been developed in the last years (Figure 1).

Most of these biomarkers are still under study and not available yet for use in clinical practice. It is aimed to develop high-specific and sensitive circulating biomarkers using DNA, RNA and a metabolomic approach. Combination markers and multianalyte analysis seem to be more effective than the current use of monoanalytes because of a higher sensitivity [28, 49, 77]. A summary of potential novel circulating and tissue biomarkers for diagnosis, prognosis, and therapy response prediction, as well as their relation with tumor localization, is shown in Figure 2.

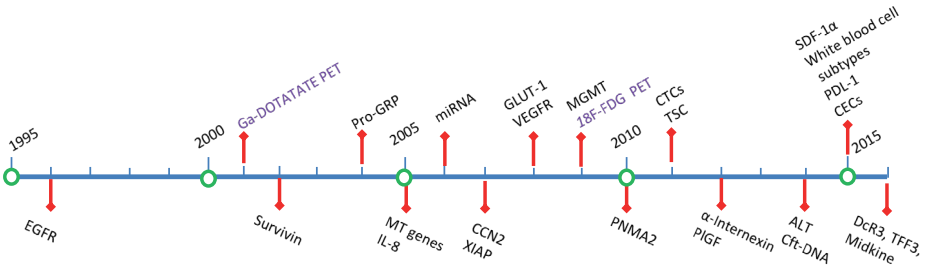


Figure 1: Timeline of the publication of potential novel biochemical and therapeutic markers in neuroendocrine tumors. Monoanalytes, transcripts, DNA-, RNA- , immune- markers are shown. They are still mostly under study and not available for use in clinical practice. Image-based modalities are represented in purple.

Legend: EGFR: epidermal growth factor receptor; proGRP: progastrin-releasing peptide; MT: multitranscript; CCN2: connective tissue growth factor for carcinoid heart disease; IL-8: interleukin 8; miRNA: microRNA; XIAP: X-linked Inhibitor of Apoptosis; GLUT-1: glucose transporters type 1; VEGFR: vascular endothelial growth factor receptor; MGMT: O-6-methylguanine-DNA methyltransferase; 18F—FDG PET: 18-fluorodeoxyglucose positron emission tomography; PNMA2: paraneoplastic Ma antigen 2; CTCs: circulating tumor cells; TSC: Tuberous sclerosis complex; PlGF: Placental growth factor; ALT: alternative lengthening of telomeres; cftDNA: circulating cell free tumor DNA; SDF-1α: stromal cell-derived factor 1α; PD-L: programmed death ligand-1; CECs: circulating endothelial cells. Imaging techniques, as reference, are presented in purple.

a. Peptides and growth factors:

Several peptides and growth factors (Table 2) have been studied for a (potential) role as biomarkers in NETs and may: (1) help to localize primary tumors (e.g. progastrin-releasing peptide in lung NETs, connective tissue growth factor (CCN2), paraneoplastic Ma antigen 2 , DcR3, TFF3, and midkine in small intestine NETs [28, 44, 46, 78]); (2) predict the outcome in functioning NETS (e.g. α-Internexin in insulinomas [79, 80]) or predict early

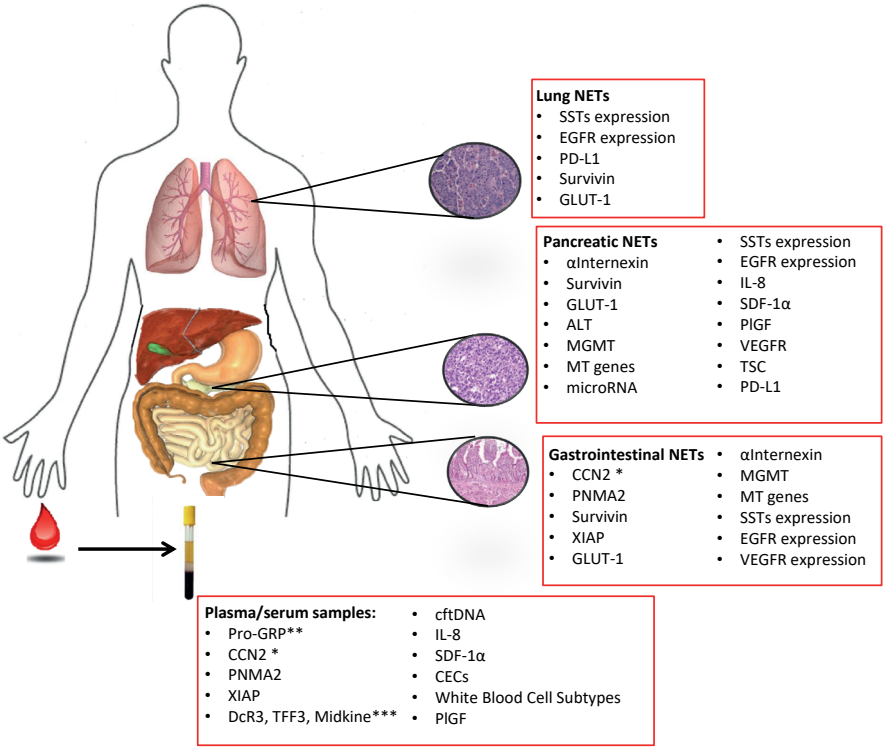


Figure 2: Summary of potential novel diagnostic and therapeutic markers in neuroendocrine tumors

Several tumor or plasma/serum biomarkers seem to play a role in the diagnosis or follow-up in lung and GEP-NETs. Its presence may be determined in serum or tissue samples.

Legend: * specific for carcinoid heart disease; ** only for lung-NETs; *** only for small intestine NETs; proGRP: progastrin-releasing peptide; CCN2: connective tissue growth factor for carcinoid heart disease; PNMA2: paraneoplastic Ma antigen 2; XIAP: X-linked Inhibitor of Apoptosis; GLUT-1: glucose transporters type 1; ALT: alternative lengthening of telomeres; MGMT: O-6-methylguanine-DNA methyltransferase; MT: multitranscript; cftDNA: circulating cell free tumor DNA; EGFR: epidermal growth factor receptor; SDF-1α: stromal cell-derived factor 1α; CECs: circulating endothelial cells; PlGF: Placental growth factor; VEGFR: vascular endothelial growth factor receptor; TSC: tuberous sclerosis complex; SSTs: somatostatin receptor; PD-L: programmed death ligand-1.

complications in patients with carcinoid heart disease [78]; and (3) add information to that provided by other circulating/tissue markers for treatment response evaluation and outcome prediction (e.g. pro-GRP and CgA for predicting outcome/therapeutic response in lung carcinoids; α-Internexin and Ki67 in insulinomas [44, 81-83] or as part of multianalyte tests [46]). Additionally, some peptides may be useful to correlate with imaging techniques. For instance, glucose transporter 1 (GLUT1) expression in NETs is associated with the Ki67 index and 18-fluorodeoxyglucose (FDG) uptake at FDG-positron emission tomography

Table 2: Peptides and growth factors as novel markers in NETs

Peptide/growth factor	Function	Potential role as marker in NETs
Progastrin-releasing peptide (proGRP)	Precursor of gastrin-releasing peptide, a neuropeptide hormone widely distributed throughout the gastrointestinal and pulmonary tract [85]	Primary tumor localization in patients with a metastatic NET of unknown origin. Complementary marker to CgA in lung NET for treatment response evaluation and survival [44, 86]
Connective tissue growth factor for carcinoid heart disease (CCN2)	CCN2 is an early gene product of the CCN family of matricellular proteins, which are involved in cell proliferation, angiogenesis, tumourigenesis and wound healing. It may be involved in the pathogenesis of carcinoid heart disease [78, 87]	Independent predictor of both reduced right ventricular function and right-sided valve regurgitation (its plasma levels are inversely related to right ventricular function levels) Early predictor of cardiac fibrosis [78]
Paraneoplastic Ma antigen 2 (PNMA2)	Antineuronal antibodies identified as markers of neurological paraneoplastic syndromes [88]	Allows the identification of almost 50% of small bowel NETs at the primary stage of the disease Correlation with disease progression and recurrence free survival [45]
α-Internexin	Cytoskeleton protein involved in tumorigenesis and disease progression [89]	Association with proliferation, ki67 index and malignancy [79]
X-linked Inhibitor of Apoptosis (XIAP)	Inhibitor of apoptotic cell death in cancer cells [90, 91]	Potential target therapies [92-94]
Glucose transporters type 1 (GLUT-1)	Mediate the transport of glucose across the cellular membrane and are commonly overexpressed in tumors, probably related with higher metabolism and cell growth [95]	Predictor of risk of death in neuroendocrine lung carcinomas and lung carcinoids [96] Relation with Ki67 index in GEP- and lung NETs [84, 97] Correlation with the uptake in 18-FDG-PET [84]
DcR3	Regulates cytokines that influence tumor growth and reduce apoptotic stimuli [98]	DcR3 correlates to liver metastasis and worse survival Predictor of treatment resistant tumors [46]
TFF3	Protects and repairs epithelial surfaces Enhances migration, angiogenesis, and inhibits apoptosis [99-101]	Higher concentrations have been correlated to reduced survival [46]
Midkine	Promotes tumor cells migration, angiogenesis and reduces apoptosis [102]	Predictive marker to chemotherapy response[103, 104]

(PET) scans [84]. GLUT-1 expression may serve as an additional marker for aggressiveness of NETs and may add to a more accurate grading [84]. Although some of these peptides have been suggested as promising biomarkers, most of them are non-specific. In addition, their applicability is limited, due to their sensitivity and specificity (Table 1) and the absence of cut-off levels. In addition, some of them have been described only in single retrospective studies, thus further validation in larger and longitudinal cohorts is still required.

b. DNA markers:

These markers are expected to improve diagnosis in NETs, especially when the primary tumor is unknown, and to predict drug response. They include the determination of mutations which are associated with alternative lengthening of telomeres [105, 106], the expression of the DNA repair enzyme O-6-methylguanine-DNA methyltransferase which may predict the clinical responses to alkylating agents including temozolamide [107, 108] and the evaluation of cell-free DNA in liquid biopsy which contains identical genetic defects as the primary tumor [109]. A schematic overview of DNA markers is depicted in Figure 3.

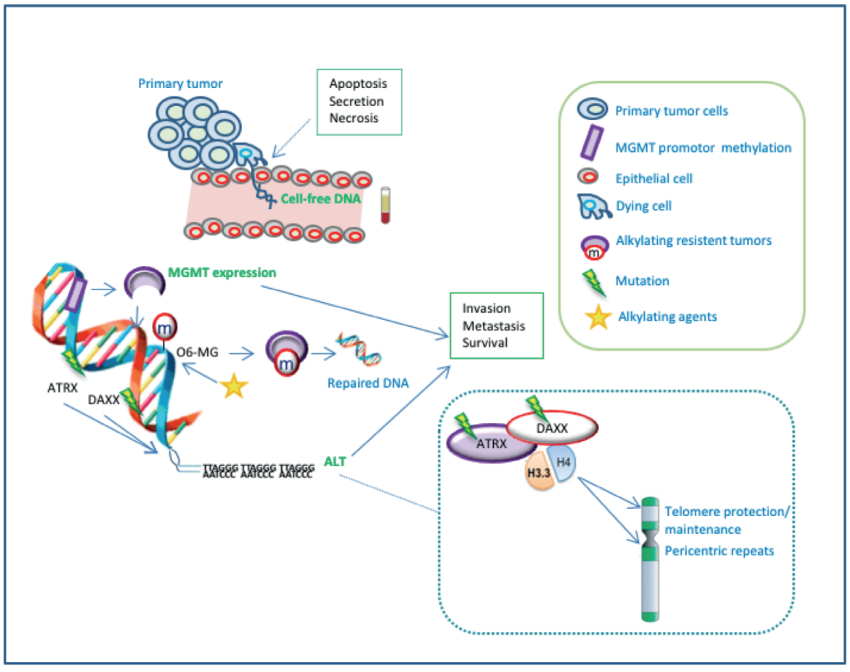


Figure 3: Summary of DNA markers in neuroendocrine tumors: DNA markers are presented in green. Tumor cells release small fragments of cftDNA into circulation by multiple mechanisms, cftDNA contains identical genetic defects compared to the primary tumor. DNA methylation at the O6 position of guanine results in apoptosis and tumor cell death; in GEP-NETs, the methylation *of MGMT* promoter and loss of MGMT protein expression have been reported. Inactivating mutations in ATRX and DAXX genes are associated with ALT.

Legend: cftDNA: cell free tumor DNA; O-6-MGMT: O-6-methylguanidine-DNA methyltransferase; O6MG: DNA methylation at the O6 position of guanine; ALT: alternative lengthening of telomeres; ATRX X linked transcriptional regulator; DAXX: death domain-associated protein 6

c. RNA markers:

These are novel and potentially promising minimally invasive markers used for diagnostic purpose and/or to identify therapeutic targets for NETs. Specifically, the identification of circulating target genes using PCR-amplification has been used for determining stage, prognosis, recurrence or new metastasis in several cancers [110-112]. Modlin and co-workers have developed a PCR-based molecular test using 51 genes for identifying GEP-NETs [49]. For this so-called NETtest, a score, based on tissue and peripheral blood transcriptomes, was developed [49] as a diagnostic and follow-up tool for NETs [48, 49, 77, 113]. NETtest results were shown to differentiate progressive disease [48] and to predict tumor response to somatostatin analogs (SSAs) [114] and peptide receptor radionuclide therapy (PRRT) [47]. Despite these promising results, prospective independent validation is desirable in order to establish the reproducibility of the results and their interpretation.

Additionally, dysregulated microRNAs (miRNA) have been correlated with diagnosis, staging, progression, prognosis and therapeutic response in several tumors, including NETs [115]. Their up- and down-regulation has been described and associated with histological characteristics (e.g. Ki-67, degree of malignancy) and prognosis characteristics, including OS [116, 117]. The therapeutic strategy for miRNAs includes the oncogenic miRNA inhibition or the introduction of a tumor suppressor miRNA [118]. However, the currently available technology is not robust enough to support its clinical use yet [119]. Furthermore, dysregulation of miRNAs is not tumor specific and the absence of cut-off levels for differentiating tissue and tumor subtypes, the lack of reproducibility in other NET cohorts and the difficulties in their interpretation, limit the clinical application of miRNAs. Further studies are required to evaluate the application of miRNAs as clinical and therapeutic markers in NETs.

d. Therapeutic/prognostic biomarkers:

Somatostatin/cortistatin system components (ligands and receptors) are widely expressed in tissues, including the gastrointestinal tract, where they inhibit endocrine secretions, motility and absorption, in a paracrine and endocrine manner [120, 121]. Somatostatin acts through the binding and activation of a family of five G-protein-coupled somatostatin receptor subtypes ($SST_{1\text{-}5}$), which are widely distributed in the organism [122-124]. SSTs are a family of 5 G-protein-coupled, 7 transmembrane domains receptors that trigger different intracellular signaling pathways. Through their activation, in addition to secretion processes, proliferation, differentiation and angiogenesis are regulated [124]. The complexity of somatostatin/cortistatin system has increased in recent years after the identification of SSTs splicing variants of the SST_5 gene (SST_5TMD_4 and SST_5TMD_5) [125-131], which may be dysregulated in tumor pathologies where they may be associated with aggressive features [128, 132].

SSAs are considered to be the preferred first-line treatment option in functionally active NETs, including those associated with the carcinoid syndrome and functional PNETs

[133, 134]. Additionally, monthly administered long-acting preparations of octreotide and lanreotide are usually used for disease stabilization in NETs [135, 136]. The effects of SSAs depends on the presence of SSTs in the tumor (octreotide and lanreotide bind preferably to SST_2 and pasireotide has high binding affinity to multiple SSTs, particularly SST_5; see also figure 5) [137].

Tightly related with the somatostatin system, the ghrelin system is involved in the regulation of multiple (patho)-physiological functions, including hormonal secretion, β-cell survival, as well as appetite and gastric motility [138-141]. The acylation of the third serine residue in ghrelin molecule is necessary for its activation, which is catalyzed by the ghrelin-O-acyl-transferase (GOAT) enzyme [141, 142]. Acylated ghrelin binds and activates its canonical ghrelin receptor, GHSR1a. Additionally, some ghrelin system variants resulting from post-transcriptional modifications or alternative splicing have been identified, including the In1-ghrelin [138, 143] and a truncated receptor GHSR1b, with unknown ligand and function [138, 143, 144].

In recent years, there is increasing interest in somatostatin/cortistatin and ghrelin systems, since alterations in some of their components seems associated with the development/progression of various cancers [143, 145-148]. Both, ghrelin [132, 149] and somatostatin systems [22, 122, 150] have been described in NETs, but the clinical-molecular correlations have not been fully elucidated [149, 151]. Their use as tissue markers may provide information about clinical evolution and outcome [132, 149]. The molecular expression and clinical relations of both systems are described in chapter 2, 3 and 4 of this thesis. In particular, SSTs expression in NETs is considered to have therapeutic implications for treatment with SSAs and for PRRT.

In recent years, the use of molecular targeted therapies has been approved in NETs. The possibility to peripherally measure monoanalytes directly related to the drug mechanism of action represents an important approach to predict treatment response. In this sense, some molecular biomarkers could play a role as prognostic markers for treatment response to tyrosine kinase- and mTOR inhibitors. For sunitinib, such biomarkers include: the epidermal growth factor receptor [152-155], vascular endothelial growth factor (VEGF) and its transmembrane receptors (VEFGR-1, VEFGR-2, VEFGR-3) [156], interleukin-8 [157], stromal cell-derived factor-1α [157] and circulating tumor, endothelial and white cells [158, 159]]. For everolimus, circulating levels of placental growth factor [160] and tuberous sclerosis complex mutations [161] have been described. Additionally, some relations were shown between these markers, tumor response, PFS and OS [158, 159]. Their use may identify patients with increased drug sensitivity and higher possibilities of drug responsiveness [161]. However, currently there is no consensus for supporting their use. Further multicenter, longitudinal studies in this field are required. A summary of the current potential tumor biomarkers for tyrosine kinase and mTOR inhibitors is shown in Figure 4.

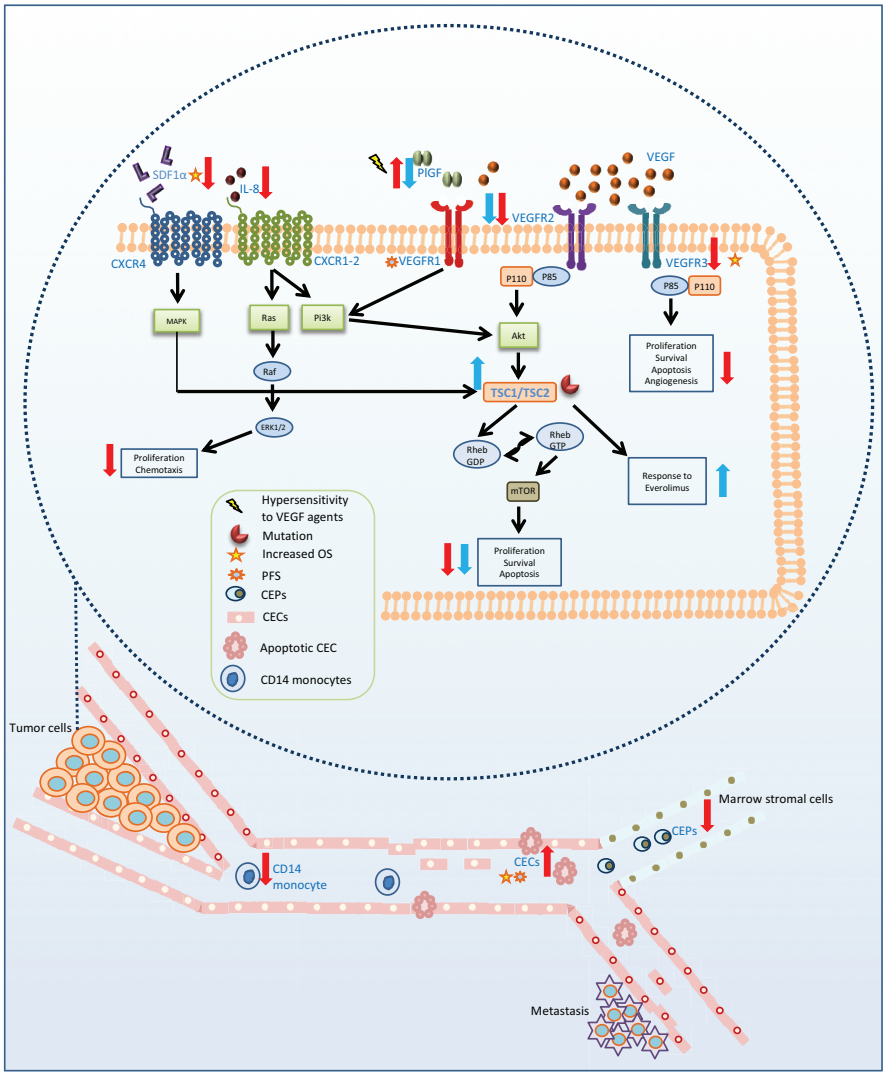


Figure 4: Therapeutic markers: molecular biomarkers for tyrosine kinase and mTOR inhibitors. Blue arrows represent the effect of the mTOR inhibitor everolimus and red arrows the effect of the tyrosine kinase inhibitor sunitinib. Molecular markers are presented in blue. Sunitinib has been related to decreased SDF1, IL-8, VEGFR 2-3, CD14 monocytes expressing VEGFR, decreased circulating endothelial precursors (CEPs), increased circulating endothelial cells (CECs) and probably decreased PlGF. Everolimus has been related to decreased PlGF and VEGFR2; additionally, mTOR directed therapies may be more effective in tumors with tuberous sclerosis complex 1 (TSC1) somatic mutation. Factors related to PFS and OS are also shown.

Legend: SDF-1α: Stromal cell-derived factor 1α; IL-8: interleukin-8; PlGF: placental growth factor; CXCR 1,2,4: chemokine family receptor 1,2,4; VEGF: vascular endothelial growth factor; VEGFR 1-3: vascular endothelial growth factor receptor 1-3; TSC 1-2: Tuberous sclerosis complex 1-2; CECs: circulating endothelial cells; CPECs: circulating endothelial precursors derived from the bone marrow.

2.3 Imaging

Tumor staging using imaging techniques should be performed in all NETs. Specifically, endoscopic evaluation in small (<1 cm) low-grade gastric- or rectal NETs is recommended [23, 162]. Most cases require evaluation with conventional imaging techniques, usually computed tomography (CT) or magnetic resonance imaging [30, 163]. SSTs-based imaging techniques help to localize primary tumor and metastasis for disease staging [164, 165]. Additionally, they help to make therapeutic decisions and are used to evaluate prognosis in NETs [165, 166]. Initially ^{111}In-DTPA-octreotide (Octreoscan®) was used. In recent years, the positron emitter ^{68}Gallium, which labels several somatostatin analogs, combined with positron emission tomography CT (^{68}Ga PET/CT), probably represents the gold standard for SSTs imaging of NETs [163, 167]. Other functional imaging techniques such as 18-fluorodeoxiglucose PET/CT (18-FDG PET/CT) have higher accuracy for poorly differentiated NETs and may be useful for evaluating atypical carcinoids. Importantly, images of 18-FDG PET/CT should be analyzed in combination with ^{68}Ga-PET/CT [163, 166, 168]. Additionally, a radiolabelled glucagon-related peptide 1 receptor agonist [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH2]-exendin-4, has been reported as a promising imaging technique for the localization of insulinomas [169].

3. Treatment

3.1 Surgery

Surgery with margin-negative resection and, in some cases, adequate lymphadenectomy is the only curative treatment for localized NETs [170]. Unfortunately, patients often present with extended or metastatic disease at diagnosis [7]. Despite this, surgical management may still be an option with tumor debulking and resection of limited liver/lymph node metastasis with the subsequent use of liver-directed therapies [171-173]. Surgery is also useful for symptom control, specifically primary tumor resection/enucleation in localized functioning NETs is indicated. In some metastasized functioning NETs, decreased tumor load after surgery improves the associated symptoms. Additionally, in patients with recurrent/severe abdominal pain due to mesenteric fibrosis, surgery may improve clinical symptoms and obstruction [171-173]. Finally, bilateral adrenalectomy in patients with ectopic ACTH syndrome should be considered in cases of uncontrollable hypercortisolism, unknown primary tumor or extended disease [174].

3.2 Medical treatment

Hormone excess symptom control

SSAs are first-line therapy in functionally active NETs, including those associated with the carcinoid syndrome and functional PNETs [133]. The mechanisms of action of current medical options for functioning NETs are depicted in Figure 5.

Medical options for functioning NETs are summarized in Table 3. Chemotherapy may also be used in some aggressive cases [133, 175]; this therapeutic option is not discussed in this thesis. Since in Part II of this thesis novel therapeutic options for ACTH and serotonin overproduction are evaluated, only carcinoid syndrome and ectopic ACTH syndrome are described in more detail.

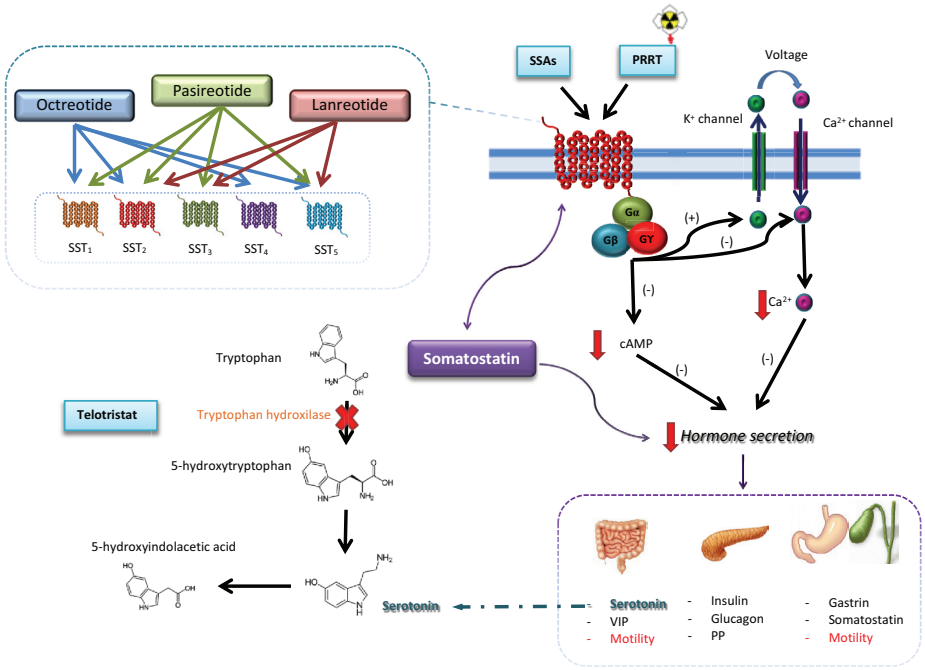


Figure 5: Current medical treatment for symptoms control in neuroendocrine tumors. Short-, long-acting and radiolabeled- somatostatin analogs bind to G-protein linked receptors on the cell surface with variable affinity. The inhibition of the cAMP and the decrease in intracellular calcium levels inhibit hormone release. Somatostatin influences hormone secretion and motility in the whole gastrointestinal tract. Serotonin production may be also decreased by telotristat, which inhibits the rate limiting step in the serotonin secretion (the enzyme tryptophan hydroxylase).
Legend: SSTs: somatostatin receptor; SSAs: somatostatin analog; PRRT: peptide receptor radionuclide therapy; cAMP: cyclic adenosine monophosphate; VIP: vasoactive intestinal peptide; PP: pancreatic polypeptide.

Table 3: Medical Treatment for functioning NETs

Functioning NET	Medical treatment	Mechanism of action/clinical relevance
Carcinoid syndrome	-SSAs, pasireotide, telotristat.	Please refer to the text for explanation of the medical options.
Insulinoma:	- Diazoxide (benzothiadiazine derivative)	- Inhibits insulin secretion, increases the hepatic glucose production and inhibits tissue glucose uptake [206-210].
	- Octreotide LAR and lanreotide autogel	- Decrease insulin release if SSTRs are expressed [209], otherwise, paradoxical decrease in blood glucose by suppressing glucagon release [208].
	- Pasireotide	- Mediates insulin secretion by binding SSTR5 [211]
	- Everolimus	- Decreases insulin release (AMP-activated protein kinase (AMPK)/c-Jun N-terminal kinase (JNK)/FoxO pathway); induce peripheral insulin resistance (glucose transporter 1 downregulation) [212-215].
	- PRRT	- Decreases insulin secretion, antitumor effect [208].
Glucagonoma:	- Octreotide LAR and lanreotide autogel	- The necrolytic migratory erythema improves despite the persistence of elevated serum glucagon levels [216]
	- Pasireotide	- Clinical response in octreotide-resistant tumors [20, 217].
	- Everolimus, sunitinib	- Clinical response after SSAs failure [218, 219].
	- PRRT with ^{90}Yttrium-DOTATOC or ^{177}Lu-DOTATATE	- Disease stabilization or tumor regression with subsequent symptoms control [220].
Gastrinoma:	- H^+-K^+-ATPase proton-pump inhibitors (PPIs)	-Control gastric hypersecretion [21]
	- SSAs	- Suppress gastrin secretion and normalizes gastric acid secretion [26–34]. Prevent the enterochromaffin-like cell hyperplasia or the development of gastric type 2 NETs [221].
	- IFN-α	- Improves clinical symptoms caused by hypergastrinemia only in stabilized tumors (41).
VIPoma, somatostatinoma:	- SSAs (Octreotide LAR and lanreotide autogel)	- Control symptoms in the majority of patients [29, 222, 223]
	- Glucocorticoids	- In SSAs refractory cases [223].
	- Molecular targeted therapy and PRRT	-In metastasized cases [218-220, 224-226].
Ectopic hormone producing syndromes:	• ***ACTH:***	- Please refer to the text for explanation of the medical options.
	• ***GHRH***:	
	-SSAs (octreotide and lanreotide)	- Low ectopic tumoral production of GHRH, with a subsequent decrease in circulating GH and insulin-like growth factor-1 (IGF-1) levels [227, 228].
	• ***PTH related protein:*** Includes hypercalcemia control	
	- Intravenous isotonic saline	- Corrects volume depletion
	- Bisphosphonates	- Interfere with the osteoclast-mediated bone resorption
	- Denosumab	- Reduces the formation, function, and survival of osteoclasts via the nuclear factor κB (RANK) pathway [190, 229, 230]
	- SSAs	- Improve symptoms control but might be insufficient in patients with tumor progression [190, 231].
	- PRRT with ^{177}Lu-DOTATATE [190].	- Tumor stabilization with parallel calcium control [190].

Carcinoid syndrome (CS)

CS is mediated by several active hormones, especially serotonin [16, 17]. The role of SSAs for improving secretory diarrhea and flushes in NETs was initially described in 1978 [176, 177]. Since then, short-acting octreotide was considered as a treatment option for carcinoid syndrome. The efficacy of short- and long-acting octreotide is similar once circulating octreotide steady-state concentrations are achieved [178, 179]. Long-acting preparations of SSAs are widely used, as these improve flushes and diarrhea in 53-75% and 45-80% of cases, respectively [180, 181]. Octreotide and long-acting lanreotide similarly reduce u5-HIAA acid and improve quality of life in NET patients [180]. Both octreotide and lanreotide are well tolerated and side effects are observed only in 14-29% of patients [180]. In addition, a favorable clinical response of carcinoid syndrome-related symptoms has also been reported in patients after treatment with PRRT [182, 183].

Pasireotide, a SSA with affinity to multiple somatostatin receptors, has been also tested in patients with octreotide-LAR resistant tumors. Here, pasireotide showed efficacy in 33% of patients when administered 150 μg twice daily, escalated to a maximum dose of 1200 μg per day [184]. α-interferon in combination with octreotide was suggested as an effective treatment for symptom control, but unfortunately the use of this combination is limited due to the high rate of adverse effects (attributed to α-interferon in 5-76% of cases) [185].

Recently, telotristat etiprate, a novel inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of serotonin, has been developed. Telotristat etiprate decreases u5-HIAA and improves CS symptoms [186]. Remarkable, published data of its *in vitro* effects is lacking. This novel drug will be extensively described in chapter 7 of this thesis.

Importantly, SSAs and/or tumor debulking techniques may improve the hemodynamic impact of tumor vasoactive agents on CHD [178, 187, 188] but there is no concluding evidence suggesting that these treatment options can stop the progression of CHD [189].

Ectopic hormone producing syndromes

Ectopic hormone production is rare in NETs. The treatment aims in these patients include symptomatic long-term control, tumor stabilization or reduction, and prolongation of (progression-free) survival [190].

Ectopic ACTH syndrome

The ectopic ACTH syndrome (EAS) causes approximately 10% of all cases of Cushing syndrome [191, 192]. Clinical evolution is usually faster and characterized by mineralocorticoid effects (hypertension, hypokalemia, and edema), thromboembolic disease and opportunistic infections [193]. Curative surgery is the primary treatment option but is often not possible [191, 194]. EAS can result in a critical condition for which aggressive medical therapy or life-saving bilateral adrenalectomy is necessary [193]. Medical treatment options for EAS include: (1) tumor-directed drugs including somatostatin analogs (octreotide, pasireotide)

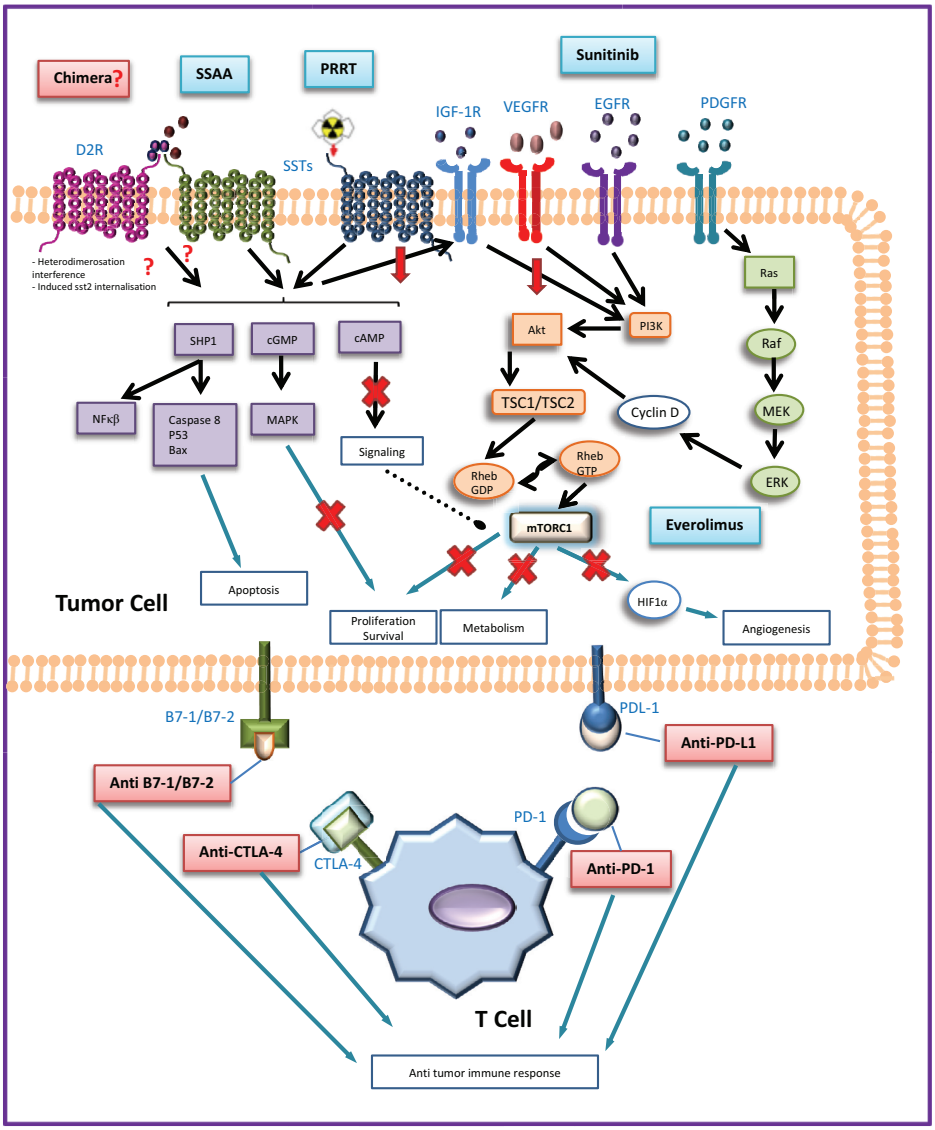


Figure 6: Current and future medical options for tumor control in neuroendocrine tumors. Current therapeutic options are presented in blue, possible novel therapeutic options are presented in red. SSAs and PRRT: increase apoptosis by activating the protein tyrosine phosphatase SHP1; decrease cell proliferation and survival through the mitogen-activated protein kinase and cyclic adenosine monophosphate; and inhibit the signaling of the insulin-like growth factor receptor type 1; additionally, PRRT produces DNA double stand breaks induced by β-irradiation, leading consequently to apoptosis. Sunitinib is a multikinase inhibitor that modulates the phosphoinositate-3-kinase/Akt pathway (it blocks the vascular endothelial growth factor receptors 1-3, the platelet-derived growth factor receptors α and β, and the epidermal growth factor receptor). Everolimus decreases tumor cell proliferation, metabolism, survival and angiogenesis through the mammalian target of rapamycin complex-1. The

indirect inhibition of mTOR through the phosphoinositate-3-kinase/Akt produced by the SSAs seems to increase sensitivity to mTOR inhibition. Multi-receptor chimeras may bind SSTs and D2R, and may enhance the signaling of the cAMP and JNK pathways; induced SST_2 internalization and SST_2 heterodimerisation interference have been also hypothesized. The interaction between some receptors expressed on the surface of cytotoxic T-cells (PD-1, CTLA-4) with ligands expressed on the tumor cells (PD-L1, B7-1/B7-2) downregulates the immune response to tumor cells; novel drugs that target these specific immune checkpoints inhibit this interaction allowing the immune system to maximize an efficient antitumor response.
Legend: SSAs: somatostatin analogs, PRRT: peptide receptor radionuclide therapy; IGF-1R: insulin-growth factor receptor type 1; VEGFR: vascular endothelial growth factor; EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptors; TSC: tuberous sclerosis complex; mTOR: mammalian Target of Rapamycin; CTL4: cytotoxic T-lymphocyte antigen-4; PD-L1: Programmed death-ligand 1.

and dopamine agonists that decrease tumoral ACTH secretion [193, 195-198]. The identification of SSTs expression in the tumor using radiolabeled somatostatin analogs, may also help to identify suitable patients that might benefit from PRRT [195, 199, 200]. In addition, the tyrosine kinase inhibitors vandetanib and sorafenib may have antisecretory effects in selected cases with EAS [201, 202]; (2) steroid synthesis inhibitors which directly suppress adrenal cortisol production. In this sense, a combination of ketoconazole, metyrapone and mitotane was shown to be effective in critically ill patients with EAS [203]. Additionally, the anaesthetic drug etomidate can also rapidly suppress cortisol levels in an ICU setting [204]; and (3) glucocorticoid receptor antagonists. Mifepristone has a short onset of action and was shown to reverse morbidity of EAS in several cases [205].

Tumor growth control

According to the latest analysis of the SEER database, 27.4% of NETs have distant metastasis at diagnosis and 20% have regional infiltration [7]. Survival in NETs is related to tumor localization, tumor load and grading and these factors should be considered when selecting the appropriate medical treatment that would allow tumor stabilization and/or shrinkage. Current and promising novel medical options for tumor growth control in NETs (excluding chemotherapy) are described in this chapter and presented in Figure 6.

The current registered clinical trials of novel and combination therapies in NETs are depicted in Table 4.

Somatostatin analogs

The antiproliferative effect of SSAs depends on the level of expression of SSTs in the tumor, although indirect antitumor effects have been described as well [232]. SSAs may inhibit the cell cycle and increase apoptosis, and indirect effects may include immuno-modulation, antiangiogenic effects, and growth factor inhibition [135, 222, 233]. Long-acting prepara-

Table 4: Registered clinical trials for tumor growth control medical therapies in NETs

Drug	Study Characteristics	Primary outcome/ ClinicalTrials.gov Identifier
Lanreotide (CLARINET FORTE)	Open label single group clinical trial for evaluating the efficacy and safety of lanreotide 120 mg every 14 days in well differentiated, metastatic or locally advanced, unresectable pancreatic or midgut NETs with radiological progression under lanreotide 120 mg every 28 days	PFS (102 weeks)/ NCT02651987
^{177}Lu-DOTA0-Tyr3-Octreotate (NETTER-1)	Multi-center, randomized, phase III study comparing ^{177}Lu-DOTA0-Tyr3-Octreotate to Octreotide LAR in patients with inoperable, progressive, somatostatin receptor positive midgut carcinoid tumors	PFS, OS data is pending/ NCT02651987
Sulfatinib	Randomized, multi-center phase III study to evaluate the efficacy and safety of sulfatinib (angio-immuno kinase inhibitor targeting VEGFR, FGFR1 and CSF-1R kinases) vs placebo in advanced PNETs	PFS (7 months after the last patient enrolled)/ NCT02589821
Sulfatinib	Randomized, double blind, multi-center phase III study to evaluate the efficacy and safety of sulfatinib vs placebo in advanced PNETs	PFS (9 months after the last patient enrolled)/ NCT02588170
177Lu-PRRT vs 177Lu-PRRT plus capecitabine	Open label phase II study to compare the efficacy of 177Lu-PRRT vs 177Lu-PRRT plus capecitabine in SSTR and 18-FDG PET/CT positive, G1-G2-G3 GEP-NETs	PFS (72 months)/NCT02736448
177 Lu-Octreotate -CAPTEM vs (i) CAPTEM and (ii) 177 Lu-Octreotate	Two parallel phase II randomized open label trials of PRRT with 177 Lu-Octreotate and CAPTEM (i) versus CAPTEM alone in the treatment of low to intermediate grade PNETs (ii) versus 177 Lu-Octreotate alone in the treatment of low to intermediate grade midgut NETs	PFS (12 months in PNETs and 24 months in midgut NETs)/ NCT02358356
177Lu-edotreotide vs Everolimus	Prospective, randomised, controlled, open-label, multicentre phase III study to evaluate efficacy and safety of PRRT with 177Lu-edotreotide compared to everolimus in GEP-NETs	PFS assessed up to 24 months/ NCT03049189
177Lu-DOTA0-Tyr3-Octreotate vs sunitinib	Open label randomized phase II antitumor efficacy of PRRT with 177Lu-DOTA0-Tyr3-Octreotate vs sunitinib in unresectable progressive well-differentiated PNETs	PFS (12 months)/NCT02230176
Everolimus and LEE011 (Recociclib)	Open label study to evaluate the efficacy and safety of the combination LEE011 (inhibitor of cyclin D1/ CDK4 and CDK6 pathway) 300 mg once daily for 3 weeks (4th week off) and everolimus 2.5 mg daily in foregut WDNETs	PFS (2 years)/NCT03070301
Everolimus and TMZ	Open label study to evaluate everolimus and temozolamide as first line treatment in advanced NEC with a Ki67 of 20-55%	Disease control rate/NCT02248012
Everolimus and bevacizumab	Randomized phase II study of everolimus alone versus combined with bevacizumab in patients with PNETs (currently active, not recruiting)	PFS (up to 3 years)/NCT01229943

Table 4 (*continued*)

Drug	Study Characteristics	Primary outcome/ ClinicalTrials.gov Identifier
Everolimus and cisplatinum	Open label phase II study of cisplatinum and everolimus in metastatic or unresectable NEC of extrapulmonary origin	Disease control rate/NCT02695459
Pembrolizumab	Open label phase 2 study of monotherapy with pembrolizumab (humanized anti-PD-1 monoclonal antibody) in patients with metastatic high-grade NETs who have failed platinum-based chemotherapy	Objective response rate/ NCT02939651
PDR001	Open label phase II study to evaluate PDR001 (high-affinity, ligand-blocking, humanized IgG4 antibody directed against PD-1) in advanced or metastatic, well-differentiated, non-functional, thoracic and GEP-NETs or GEP-NECs	Overall response rate/NCT02955069
Durvalumab and tremelimumab	Multi-center open label phase II study to evaluate the combination therapy between durvalumab (MEDI4736; humanized antibody against PD-1) and tremelimumab (CTLA-4 inhibitor) in advanced/ metastatic, grade 1/2 (G1/G2) lung and GEP-NETs, and grade 3 (G3) GEP- tumors or of unknown primary site after progression to previous therapies.	Clinical benefit rate/NCT03095274

Legend: PRRT: peptide receptor radionuclide therapy; CAPTEM: Capecitabine/temozolamide; PFS: progression free survival; OS: overall survival; VEGFR: vascular endothelial growth factor; FGFR : fibroblast growth factor receptor; CSF-1R: colony stimulating factor 1 receptor; PNETs: pancreatic neuroendocrine tumors; PRRT: peptide receptor radionuclide therapy; 18-FDG PET/CT:18-fluorodeoxiglucose positron emission tomography – computed tomography; GEP-NET: gastroenteropancreatic neuroendocrine tumors; CDK: cyclin-dependent kinases; WDNETs: well differentiated neuroendocrine tumors; TMZ: Temozolamide; NEC: neuroendocrine carcinoma; PDGFR: platelet-derived growth factor receptor; PD1: programmed death-1; NECs: neuroendocrine carcinomas; CTLA-4 :cytotoxic T-lymphocyte-associated protein 4.

tions of octreotide and lanreotide are usually used for disease stabilization in NETs [135, 136]. The anti-proliferative effect of SSAs in NETs was initially evaluated in the PROMID study [234]. In this phase III B study, 85 well-differentiated metastatic midgut NETs were included. Patients randomly received placebo or octreotide-LAR 30 mg every four weeks. A difference of 8.3 months in tumor progression was observed after comparing the octreotide and the placebo group. Stable disease after six months was observed in 66.7% of patients treated with octreotide-LAR, compared to 37.2% in the placebo group [234]. Despite the initial good response to octreotide LAR, the results from the long-term survival analysis revealed that the overall survival (OS) was not significantly different in the placebo and in the octreotide group [235]. Similar to the PROMID study, the CLARINET study revealed that lanreotide Autogel (120 mg every 28 days) increased progression free survival (PFS) of patients with metastatic well- and moderate-differentiated gastroenteropancreatic NETs when compared to placebo [(PFS rate of 65.1% in the lanreotide group and 33% in the placebo group) [236]]. Usually SSAs induce tumor stabilization but in selected cases SSAs can cause tumor shrinkage, possibly due to their effects on the perfusion of liver metastases [237]. Currently, the CLARINET FORTE study is evaluating the safety and anti-tumor efficacy of lanreotide autogel 120 mg every 14 days in patients with pancreatic- or midgut NETs with progressive disease under regular dose of long-acting SSAs (NCT02651987). Pasireotide has been also studied in NETs, in which pasireotide concentrations correlated with tumor shrinkage in a non-significant manner [238]. Other studies have reported predominantly disease stabilization (60%) in treatment-naïve patients with grade 1-2 NETs, but also partial response (4%) and disease progression (36%) have been reported [239]. Additionally, pasireotide-LAR has been compared to octreotide-LAR in patients with metastatic NETs and carcinoid symptoms. In these patients, pasireotide tended to increase the tumor control rate after six months and was associated with a longer PFS [240]. In the phase 2 prospective LUNA study in advanced (unresectable or metastatic), progressive, well differentiated carcinoid tumors of the lung or thymus, pasireotide LAR treatment resulted in an objective tumor response in 39% of patients [241]. In a randomized, open-label, phase 2 study of everolimus in combination with pasireotide LAR or everolimus alone in advanced, well-differentiated, progressive pancreatic neuroendocrine tumors (COOPERATE-2 trial), the addition of pasireotide to everolimus was not associated with the improvement in PFS compared with everolimus alone [242]. Further investigation to evaluate the applicability of pasireotide alone or in combination with other therapies is required.

Interpheron-alpha

Interferon-alpha has antiproliferative, pro-apoptotic, cytotoxic/cytostatic and immunomodulatory effects in NETs [243, 244]. It has been considered as a second-line therapeutic option in progressive NETs under SSAs [133, 245]. Tumor response rates of about 10% have been reported [246] and its efficacy is similar to other agents, including bevacizumab,

when combined with SSAs [247]. Unfortunately, several adverse effects have been described; a pegylated formulation seems to be associated with fewer side effects, and its combination with octreotide seems to be better tolerated [246, 248]. Despite this, the availability of novel therapeutic options with higher efficacy and lower side effects, limits the applicability of this drug for tumor control [249].

Peptide receptor radionuclide therapy

PRRT with SSAs allows targeted delivery of radionuclides to tumor cells expressing high levels of SSTs. Treatment response is directly related to the expression of SSTs in the tumor, making it a predictive marker of response [165]. Tumor response may also differ according to the primary tumor localization and tumor load [250]; OS is also different in NETs of different localization [pancreas: 71 months, CI 56-86), midgut: 60 months (95% CI52-68) [251]]. In contrast, response rates are decreased in patients with larger tumor load and higher liver infiltration [252]. The pivotal phase III NETTER-1 trial for the first time evaluated the efficacy of PRRT with ^{177}Lu-DOTATATE in a multi-center, randomized clinical trial. This study included 229 patients with well-differentiated, metastatic midgut NETs that were progressive on a standard dose of long-acting SSA. Patients were randomized to receive 4 cycles of PRRT with ^{177}Lu-DOTATATE or a double dose of octreotide LAR. The primary outcome was an increase in PFS (median not reached vs 8.4 months) in favor of patients treated PRRT. This study also reported a 79% reduction in risk of progression or death compared to octreotide and an increased overall response ratio (ORR) in the PRRT group (18%) compared to 3% in the control group [253]. In those cases with tumor progression after an initial good response, retreatment with PRRT represents an alternative. In this sense, disease control rates of 70-85% have been reported, but tumor response is limited [254, 255]. ^{177}Lu-DOTATATE has been also evaluated with radiosensitizing agents; its use in combination with 5-fluorouracil, capecitabine or temozolamide may increase the response rate (ORR 24-38%), but toxicity should still be evaluated [256-258]. Similar ORR has been reported when combined to everolimus [259]. Some case reports and series have suggested the use of pre-operative PRRT for downstaging NETs [260-262], but further investigation is still required on the efficacy of neoadjuvant PRRT in patients with initially unresectable NETs. Soon the results of the COMPETE study will be available. The aim of this multi-centre phase III study is to evaluate the efficacy and safety of PRRT (^{177}Lu-Edotreotide) compared to everolimus in progressive gastroenteropancreatic- (GEP-) NETs with positive expression of SSTs (NCT03049189). Hopefully this comparison will provide information about the treatment sequence that should be followed in progressive NETs under SSAs. A representative example of tumor response to PRRT is depicted in Figure 7.

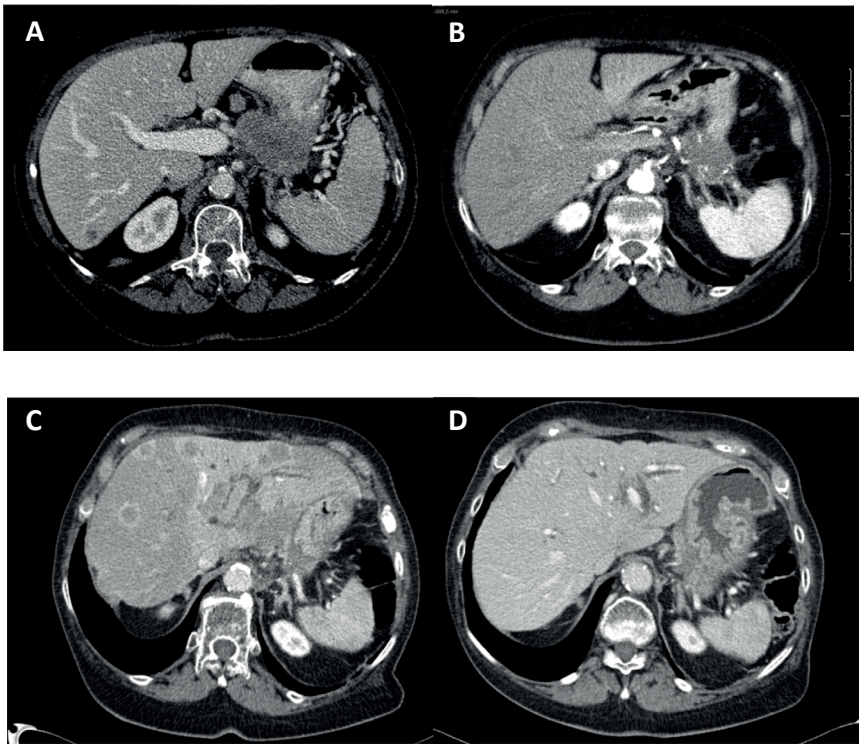


Figure 7: Peptide receptor radionuclide therapy in NETs. (A) CT imaging of a pancreas neuroendocrine tumor grade 2 with lymphatic and liver metastasis (segment 6); in this case, 4 cycles of peptide receptor radionuclide therapy (cumulative dosis 30 Gbq) was administered resulting in decreased size of the primary tumor (**B**). After 6 years, of partial response and stable disease, the primary tumor increased in size accompanied by new liver and mesenteric metastasis (**C**). Due to a first good treatment response, 2 cycles of PRRT (14.9 GBq) were administered, decreased size of primary tumor and liver metastasis were observed (**D**).

Molecular Targeted Therapy

- ***Everolimus:*** The mammalian target of rapamycin (mTOR1) pathway plays an important role in the regulation of cell proliferation in NETs [263]. The efficacy of the PI3K/AKT/mTOR inhibitor everolimus in well-differentiated NETs has been shown in several clinical trials [133]. In the phase III RADIANT 3 trial PFS was longer in the everolimus group compared to placebo [264], and its effect on PFS and OS was independent of the prior use of chemotherapy or SSAs [264-266]. Increased PFS and higher disease control rate were also reported in the RADIANT 4 study [267]. However, although everolimus is considered a safe drug, treatment can be accompanied by grade 3 and 4 drug-related adverse events (diarrhea, infections, anemia, fatigue, hyperglycemia) [267], which may limit the treatment tolerance and consequently the patient adherence. Importantly, the RADIANT 4 study,

as the previous ones, failed to demonstrate statistically significant improvements in OS [267], which should be considered especially in those patients with poor treatment tolerance. The combination of everolimus and octreotide LAR improved PFS in lung [268] and colorectal NETs [269]. Everolimus is considered as first-line therapy in progressive atypical lung carcinoids, SSTs-negative lung NETs, and in well-differentiated midgut SSTs-negative NETs [133]. Currently, several studies are evaluating the combination of everolimus with other therapies including, chemotherapeutic agents, SSAs, molecular targeted therapies, radiotherapy and PRRT.

-***Sunitinib:*** Sunitinib is as an oral multi-targeted tyrosine kinase inhibitor (TKI) that inhibits multiple angiogenic factors, including the vascular endothelial growth factor receptors 1-3 (VEGFR), the stem-cell factor receptor, and the platelet-derived growth factor receptors [270]. Increased PFS in progressive PNETs compared to placebo has been reported in those patients treated with sunitinib (SUN 1111 trial) [218], but as for everolimus, significant improvement in OS has not been reported yet.

Other therapeutic targets

The comprehensive evaluation of signaling pathways regulating cell proliferation involved in NET development and progression has opened new perspectives for the medical treatment of these tumors. mTOR inhibitors and TKIs are the most representative examples, but other novel pathway-directed therapeutic compounds are also currently evaluated in (pre-)clinical studies [271]. Other examples of potential new treatment options include immunotherapy and somatostatin-dopamine multi-receptor chimeras. The mechanisms of action of immune checkpoint inhibitors and multi-receptor chimeras are depicted in Figure 6.

Immune checkpoint inhibitors have rapidly advanced and improved the management of several tumors in the last years [272, 273]. Programmed death-ligand 1 (PD-L1) is expressed on several cancer cells and interacts with PD1, which is expressed on T cells. This ligand-receptor interaction inhibits T cells and blocks the antitumor immune response [274, 275]. The expression of PD-L1 was demonstrated in GEP- and lung (large cell neuroendocrine carcinoma) NETs [274, 276], and has been associated with clinical variables including, histological type, tumor grade, and survival [274, 277]. The expression of PD-1 and PD-L1 has also been suggested as an independent survival prognostic factor in NETs [277]. Despite immunotherapy has an important role in the management of other types of cancer, the effect on well-differentiated NETs, according to preliminary data, seems to be limited although it may represent an option for G3NETs/NECs which needs further investigation [278].

Furthermore, multi-receptor interaction has been suggested as an efficacious and selective therapeutic strategy for enhancing the effects of somatostatin [279]. The presence of hetero-dimers has been described among SSTs and between SSTs and other receptor families, including dopamine receptors, especially the dopamine receptor subtype 2 (D2R)

[280, 281]. Based on this, some structural chimeric molecules that combine elements of SSAs and dopamine analogs (DA) were developed [279]. *In vitro* studies using GEP-NET primary cultures, revealed inhibitory properties of chimeras on hormone secretion [282]. Importantly, BIM-23A760, a chimeric compound that activates SST_2 and D2R, acutely decreased growth hormone and prolactin secretion in pituitary tumors, but long-term effects disappeared due to a dopaminergic metabolite that may interfere with the activity of the parent molecule [279]. Multi-receptor targeting drugs are described in chapter 6 of this thesis.
Finally, other therapeutic options may have additional effects on cell proliferation and secretion in NETs. In this sense, ketoconazole is a steroidogenesis inhibitor which is widely used for medical treatment of Cushing Syndrome, since it improves clinical signs, symptoms and comorbidities [205]. Ketoconazole impairs adrenal and gonadal steroidogenesis by inhibiting side-chain cleavage, 17,20-lyase, and 11-β hydroxylase enzymes [283]. This drug could exert additive effects in the control of patients with severe hypercortisolemia [283]. Specifically, a direct effect ketoconazole on tumoral ACTH secretion has been suggested [284, 285] due to prolonged remission of hypercortisolemia in EAS patients [284-286] and reduced ACTH *in vitro* secretion [287]. Additionally, cytotoxic effects [288], ketoconazole-induced apoptosis [289] and changes in cell cycle phases have been described [290].
A putative association between treatment with metformin and cancer prevention/treatment is suggested [291]. Epidemiological studies have suggested a decreased risk for pancreas, liver, colon, lung, and breast cancer in patients with diabetes treated with metformin [292-295]. This protective effect of metformin has been also described in several meta-analysis [295-297]. Moreover, biguanides can inhibit cell proliferation *in vitro* in several cancer cell lines, including pancreatic and neuroendocrine tumor cells [298, 299]. Metformin stimulates the AMP-activated protein kinase (AMPK), which reduces hepatic gluconeogenesis/glycogenolysis and increases glucose uptake in the muscle [300, 301]. Additionally, it suppresses the mTOR1 pathway, reduces the insulin/insulin like growth factor 1 (IGF-1) signaling [302, 303] and mediates cell cycle arrest and apoptosis [304, 305]. Some of these actions may be also exerted in an AMPK-independent manner [306]. Closely related to metformin, statins are also commonly used in patients with metabolic syndrome or T2DM. Statins not only affect the rate limiting step in cholesterol synthesis, they also exert other clinical effects related with immunomodulatory mechanisms [307]. Additionally, some antitumor effects have been described in several tumor types, including melanoma, colon and breast cancer [308-311]. The antitumor mechanisms of statins may include induced cell-cycle arrest, apoptosis induction, decreased invasion/metastasis capacity and decreased *Ki67* expression [310-314]. In this context, these drugs are described in chapters 5 (ketoconazole) and 8 (metformin and statins).

3.3 Liver directed therapies

Non-surgical liver directed therapies may represent a primary treatment option, especially in functioning metastasized NETs. Radiofrequency ablation, cryoablation and microwave ablation are some options for small liver lesions [315]. Ablation is useful in cases of intrahepatic disease recurrence with limited liver surgical options, and as an adjuvant therapy to surgical resection in metastatic disease [316].

Since metastasis in NETs are highly vascular, and the hepatic artery supplies the majority of their blood, endovascular procedures are useful in several NETs with liver metastasis [317]. Bland embolization, chemoembolization, or radioembolization are recognized as a palliative treatment in hepatic-predominant metastatic NET patients who are not candidates for surgical resection [318]. Arterial directed interventions produce local effects and could deliver high chemotherapy doses or selective internal radiotherapy for symptomatic control of hormone release and tumor size [319]. Currently no modality has demonstrated to be superior to the others, but unfortunately, prospective, randomized, placebo-controlled studies are not available yet [319].

AIMS AND OUTLINE OF THIS THESIS

The general aims of the studies presented in this thesis are:

1. To identify potential novel tissue biomarkers for lung carcinoids and GEP-NETs
2. To evaluate the antitumor effect of registered drugs (for other medical purposes) in NETs
3. To evaluate the effects of novel drugs on NET hormone release and cell proliferation

Specifically, we studied the potential applicability of ghrelin and somatostatin systems as biomarkers in tissue samples of lung carcinoids and GEP-NETs. As registered drugs for other medical purposes, we evaluated the antitumor effects of ketoconazole, metformin and statins. Finally, somatostatin-dopamine receptor chimeras and telotristat were studied as novel drugs for hormone release and cell growth control.

Chapter 1 gives an overview of the current literature on epidemiological and clinical characteristics of NETs, diagnostic strategies and therapeutic options. Diagnosis is especially focused on novel circulating and (some) tissue biomarkers. **Part I** is focused on potential novel tissue biomarkers in NETs. **Chapter 2** describes the molecular and immunohistological presence of somatostatin/cortistatin, and ghrelin system components in human lung carcinoids, as well as their clinical and histological relations. In **chapter 3** the mRNA expression of somatostatin/cortistatin, system components in GEP-NETs is described and correlated with clinical features, histology and immunohistochemistry. Additionally, the *in vitro* evaluation of the observed clinical relation is also included. Finally, **chapter 4** describes the potential role of ghrelin O-acyltransferase (GOAT) and ghrelin receptor (GHSR1a) as

tissue markers in GEP-NETs. **Part II** is focused on novel therapeutic options in NETs. To this aim, in **chapter 5** the *in vitro* direct and indirect effects of ketoconazole in ACTH- and non-ACTH producing tumor cells are studied. Additionally, an *in vitro* pancreas model of NETs using two-dimensional and three-dimensional culture systems is extensively evaluated in **chapter 6**, and in the same chapter the *in vitro* effect of somatostatin/dopamine agonists and somatostatin-dopamine multi-receptor targeting drugs is described. Furthermore, the first report of the *in vitro* effects of the novel serotonin syntesis inhibitor telotristat is described in **chapter 7**. The clinical relation between metabolic syndrome and NETs is described in **chapter 8**, as well as some clinical data and *in vitro* effects of biguanides and statins in NETs. Finally, **chapter 9** and **10** provide a general discussion and summary of the presented data.

REFERENCES:

1. Ameri, P. and D. Ferone, *Diffuse endocrine system, neuroendocrine tumors and immunity: what's new?* Neuroendocrinology, 2012. **95**(4): p. 267-76.
2. Rosai, J., *The origin of neuroendocrine tumors and the neural crest saga.* Mod Pathol, 2011. **24 Suppl 2**: p. S53-7.
3. Barakat, M.T., K. Meeran, and S.R. Bloom, *Neuroendocrine tumours.* Endocr Relat Cancer, 2004. **11**(1): p. 1-18.
4. Xavier, S., B. Rosa, and J. Cotter, *Small bowel neuroendocrine tumors: From pathophysiology to clinical approach.* World J Gastrointest Pathophysiol, 2016. **7**(1): p. 117-24.
5. Niederle, M.B., et al., *Gastroenteropancreatic neuroendocrine tumours: the current incidence and staging based on the WHO and European Neuroendocrine Tumour Society classification: an analysis based on prospectively collected parameters.* Endocr Relat Cancer, 2010. **17**(4): p. 909-18.
6. Naalsund, A., et al., *Carcinoid lung tumors--incidence, treatment and outcomes: a population-based study.* Eur J Cardiothorac Surg, 2011. **39**(4): p. 565-9.
7. Dasari, A., et al., *Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States.* JAMA Oncol, 2017. **3**(10): p. 1335-1342.
8. Dasari, A., et al., *Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States.* JAMA Oncol, 2017.
9. Modlin, I.M., et al., *Chromogranin A--biological function and clinical utility in neuro endocrine tumor disease.* Ann Surg Oncol, 2010. **17**(9): p. 2427-43.
10. Sadowski, S.M., et al., *Prospective Study of 68Ga-DOTATATE Positron Emission Tomography/Computed Tomography for Detecting Gastro-Entero-Pancreatic Neuroendocrine Tumors and Unknown Primary Sites.* J Clin Oncol, 2016. **34**(6): p. 588-96.
11. Lawrence, B., et al., *The epidemiology of gastroenteropancreatic neuroendocrine tumors.* Endocrinol Metab Clin North Am, 2011. **40**(1): p. 1-18, vii.
12. Pape, U.F., et al., *Survival and clinical outcome of patients with neuroendocrine tumors of the gastroenteropancreatic tract in a german referral center.* Ann N Y Acad Sci, 2004. **1014**: p. 222-33.
13. Yao, J.C., et al., *One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States.* J Clin Oncol, 2008. **26**(18): p. 3063-72.
14. Veenendaal, L.M., et al., *Liver metastases of neuroendocrine tumours; early reduction of tumour load to improve life expectancy.* World J Surg Oncol, 2006. **4**: p. 35.
15. Mougey A., A.D., *Neuroendocrine Tumors: Review and Clinical Update.* Hospital Physician, 2007(51): p. 12-20.
16. Kulke, M.H., et al., *Neuroendocrine tumors, version 1.2015.* J Natl Compr Canc Netw, 2015. **13**(1): p. 78-108.
17. Rorstad, O., *Prognostic indicators for carcinoid neuroendocrine tumors of the gastrointestinal tract.* J Surg Oncol, 2005. **89**(3): p. 151-60.
18. Grozinsky-Glasberg, S., A.B. Grossman, and D.J. Gross, *Carcinoid Heart Disease: From Pathophysiology to Treatment--'Something in the Way It Moves'.* Neuroendocrinology, 2015. **101**(4): p. 263-73.
19. Matej, A., H. Bujwid, and J. Wronski, *Glycemic control in patients with insulinoma.* Hormones (Athens), 2016. **15**(4): p. 489-499.
20. John, A.M. and R.A. Schwartz, *Glucagonoma syndrome: a review and update on treatment.* J Eur Acad Dermatol Venereol, 2016. **30**(12): p. 2016-2022.

21. Jensen, R.T., *Gastrinomas: advances in diagnosis and management.* Neuroendocrinology, 2004. **80 Suppl 1**: p. 23-7.
22. Herrera-Martinez, A.D., et al., *The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features.* Lung Cancer, 2017. **109**: p. 128-136.
23. Hofland, J., et al., *Recent developments in the diagnosis and therapy of well-differentiated neuroendocrine tumours.* Neth J Med, 2018. **76**(3): p. 100-108.
24. Oberg, K.E., *Gastrointestinal neuroendocrine tumors.* Ann Oncol, 2010. **21 Suppl 7**: p. vii72-80.
25. Klimstra, D.S., et al., *The pathologic classification of neuroendocrine tumors: a review of nomenclature, grading, and staging systems.* Pancreas, 2010. **39**(6): p. 707-12.
26. Rindi, G., et al., *Grading the neuroendocrine tumors of the lung: an evidence-based proposal.* Endocr Relat Cancer, 2014. **21**(1): p. 1-16.
27. Chai, S.M., I.S. Brown, and M.P. Kumarasinghe, *Gastroenteropancreatic neuroendocrine neoplasms: selected pathology review and molecular updates.* Histopathology, 2018. **72**(1): p. 153-167.
28. Oberg, K., et al., *Consensus on biomarkers for neuroendocrine tumour disease.* Lancet Oncol, 2015. **16**(9): p. e435-46.
29. Oberg, K., et al., *Neuroendocrine gastro-entero-pancreatic tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2012. **23 Suppl 7**: p. vii124-30.
30. Niederle, B., et al., *ENETS Consensus Guidelines Update for Neuroendocrine Neoplasms of the Jejunum and Ileum.* Neuroendocrinology, 2016. **103**(2): p. 125-38.
31. O'Toole, D., R. Kianmanesh, and M. Caplin, *ENETS 2016 Consensus Guidelines for the Management of Patients with Digestive Neuroendocrine Tumors: An Update.* Neuroendocrinology, 2016. **103**(2): p. 117-8.
32. Bajetta, E., et al., *Chromogranin A, neuron specific enolase, carcinoembryonic antigen, and hydroxyindole acetic acid evaluation in patients with neuroendocrine tumors.* Cancer, 1999. **86**(5): p. 858-65.
33. Duque, M., et al., *Biomarkers in neuroendocrine tumors.* JOP, 2013. **14**(4): p. 372-6.
34. Schurmann, G., et al., *Serum chromogranin A in the diagnosis and follow-up of neuroendocrine tumors of the gastroenteropancreatic tract.* World J Surg, 1992. **16**(4): p. 697-701; discussion 701-2.
35. Seregni, E., et al., *Clinical significance of blood chromogranin A measurement in neuroendocrine tumours.* Ann Oncol, 2001. **12 Suppl 2**: p. S69-72.
36. Wang, Y.H., et al., *Chromogranin A as a marker for diagnosis, treatment, and survival in patients with gastroenteropancreatic neuroendocrine neoplasm.* Medicine (Baltimore), 2014. **93**(27): p. e247.
37. Nolting, S., et al., *Chromogranin a as serum marker for gastroenteropancreatic neuroendocrine tumors: a single center experience and literature review.* Cancers (Basel), 2012. **4**(1): p. 141-55.
38. Oberg, K., et al., *ENETS Consensus Guidelines for Standard of Care in Neuroendocrine Tumours: Biochemical Markers.* Neuroendocrinology, 2017. **105**(3): p. 201-211.
39. Zandee, W.T., et al., *Limited value for urinary 5-HIAA excretion as prognostic marker in gastrointestinal neuroendocrine tumours.* Eur J Endocrinol, 2016. **175**(5): p. 361-6.
40. Metz, D.C. and R.T. Jensen, *Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors.* Gastroenterology, 2008. **135**(5): p. 1469-92.

41. Panzuto, F., et al., *Utility of combined use of plasma levels of chromogranin A and pancreatic polypeptide in the diagnosis of gastrointestinal and pancreatic endocrine tumors.* J Endocrinol Invest, 2004. **27**(1): p. 6-11.
42. Baudin, E., et al., *Neuron-specific enolase and chromogranin A as markers of neuroendocrine tumours.* Br J Cancer, 1998. **78**(8): p. 1102-7.
43. Modlin, I.M., L. Bodei, and M. Kidd, *Neuroendocrine tumor biomarkers: From monoanalytes to transcripts and algorithms.* Best Pract Res Clin Endocrinol Metab, 2016. **30**(1): p. 59-77.
44. Korse, C.M., et al., *An elevated progastrin-releasing peptide level in patients with well-differentiated neuroendocrine tumours indicates a primary tumour in the lung and predicts a shorter survival.* Ann Oncol, 2011. **22**(12): p. 2625-30.
45. Cui, T., et al., *Paraneoplastic antigen Ma2 autoantibodies as specific blood biomarkers for detection of early recurrence of small intestine neuroendocrine tumors.* PLoS One, 2010. **5**(12): p. e16010.
46. Edfeldt, K., et al., *DcR3, TFF3 and Midkine are Novel Serum Biomarkers in Small Intestinal Neuroendocrine Tumors.* Neuroendocrinology, 2016.
47. Bodei, L., et al., *Measurement of circulating transcripts and gene cluster analysis predicts and defines therapeutic efficacy of peptide receptor radionuclide therapy (PRRT) in neuroendocrine tumors.* Eur J Nucl Med Mol Imaging, 2016. **43**(5): p. 839-51.
48. Kidd, M., I. Drozdov, and I. Modlin, *Blood and tissue neuroendocrine tumor gene cluster analysis correlate, define hallmarks and predict disease status.* Endocr Relat Cancer, 2015. **22**(4): p. 561-75.
49. Modlin, I.M., I. Drozdov, and M. Kidd, *The identification of gut neuroendocrine tumor disease by multiple synchronous transcript analysis in blood.* PLoS One, 2013. **8**(5): p. e63364.
50. Eskeland, N.L., et al., *Chromogranin A processing and secretion: specific role of endogenous and exogenous prohormone convertases in the regulated secretory pathway.* J Clin Invest, 1996. **98**(1): p. 148-56.
51. D'Amico M, A., et al., *Biological function and clinical relevance of chromogranin A and derived peptides.* Endocr Connect, 2014. **3**(2): p. R45-54.
52. Arnold, R., et al., *Plasma chromogranin A as marker for survival in patients with metastatic endocrine gastroenteropancreatic tumors.* Clin Gastroenterol Hepatol, 2008. **6**(7): p. 820-7.
53. Zatelli, M.C., et al., *Chromogranin A as a marker of neuroendocrine neoplasia: an Italian Multicenter Study.* Endocr Relat Cancer, 2007. **14**(2): p. 473-82.
54. Nehar, D., et al., *Interest of Chromogranin A for diagnosis and follow-up of endocrine tumours.* Clin Endocrinol (Oxf), 2004. **60**(5): p. 644-52.
55. Stivanello, M., et al., *Circulating chromogranin A in the assessment of patients with neuroendocrine tumours. A single institution experience.* Ann Oncol, 2001. **12 Suppl 2**: p. S73-7.
56. Walter, T., et al., *Is the combination of chromogranin A and pancreatic polypeptide serum determinations of interest in the diagnosis and follow-up of gastro-entero-pancreatic neuroendocrine tumours?* Eur J Cancer, 2012. **48**(12): p. 1766-73.
57. Ardill, J.E. and T.M. O'Dorisio, *Circulating biomarkers in neuroendocrine tumors of the enteropancreatic tract: application to diagnosis, monitoring disease, and as prognostic indicators.* Endocrinol Metab Clin North Am, 2010. **39**(4): p. 777-90.
58. Cimitan, M., et al., *Somatostatin receptor scintigraphy versus chromogranin A assay in the management of patients with neuroendocrine tumors of different types: clinical role.* Ann Oncol, 2003. **14**(7): p. 1135-41.

59. Kalkner, K.M., et al., *Somatostatin receptor scintigraphy in patients with carcinoid tumors: comparison between radioligand uptake and tumor markers.* Cancer Res, 1995. **55**(23 Suppl): p. 5801s-5804s.
60. Namwongprom, S., et al., *Correlation of chromogranin A levels and somatostatin receptor scintigraphy findings in the evaluation of metastases in carcinoid tumors.* Ann Nucl Med, 2008. **22**(4): p. 237-43.
61. Baudin, E., et al., *Impact of chromogranin A measurement in the work-up of neuroendocrine tumors.* Ann Oncol, 2001. **12 Suppl 2**: p. S79-82.
62. Tomassetti, P., et al., *Diagnostic value of plasma chromogranin A in neuroendocrine tumours.* Eur J Gastroenterol Hepatol, 2001. **13**(1): p. 55-8.
63. Nikou, G.C., et al., *Chromogranin a levels in diagnosis, treatment and follow-up of 42 patients with non-functioning pancreatic endocrine tumours.* Pancreatology, 2008. **8**(4-5): p. 510-9.
64. Ferrari, L., et al., *Accuracy and clinical correlates of two different methods for chromogranin A assay in neuroendocrine tumors.* Int J Biol Markers, 2004. **19**(4): p. 295-304.
65. Jorgensen, L.G., et al., *Serum neuron specific enolase (S-NSE) reference interval evaluation by time-resolved immunofluorometry compared with a radioimmunoassay.* Clin Chim Acta, 1996. **249**(1-2): p. 77-91.
66. Isgro, M.A., P. Bottoni, and R. Scatena, *Neuron-Specific Enolase as a Biomarker: Biochemical and Clinical Aspects.* Adv Exp Med Biol, 2015. **867**: p. 125-43.
67. van Adrichem, R.C., et al., *Serum neuron-specific enolase level is an independent predictor of overall survival in patients with gastroenteropancreatic neuroendocrine tumors.* Ann Oncol, 2016. **27**(4): p. 746-7.
68. Grouzmann, E., et al., *Neuropeptide Y and neuron-specific enolase levels in benign and malignant pheochromocytomas.* Cancer, 1990. **66**(8): p. 1833-5.
69. Nobels, F.R., et al., *Chromogranin A as serum marker for neuroendocrine neoplasia: comparison with neuron-specific enolase and the alpha-subunit of glycoprotein hormones.* J Clin Endocrinol Metab, 1997. **82**(8): p. 2622-8.
70. Yao, J.C., et al., *Chromogranin A and neuron-specific enolase as prognostic markers in patients with advanced pNET treated with everolimus.* J Clin Endocrinol Metab, 2011. **96**(12): p. 3741-9.
71. Bhattacharyya, S., et al., *Usefulness of N-terminal pro-brain natriuretic peptide as a biomarker of the presence of carcinoid heart disease.* Am J Cardiol, 2008. **102**(7): p. 938-42.
72. Korse, C.M., et al., *Chromogranin-A and N-terminal pro-brain natriuretic peptide: an excellent pair of biomarkers for diagnostics in patients with neuroendocrine tumor.* J Clin Oncol, 2009. **27**(26): p. 4293-9.
73. Korse, C.M., et al., *Chromogranin A as an alternative to 5-hydroxyindoleacetic acid in the evaluation of symptoms during treatment of patients with neuroendocrine Tumors.* Neuroendocrinology, 2009. **89**(3): p. 296-301.
74. Zuetenhorst, J.M. and B.G. Taal, *Metastatic carcinoid tumors: a clinical review.* Oncologist, 2005. **10**(2): p. 123-31.
75. Janson, E.T., et al., *Carcinoid tumors: analysis of prognostic factors and survival in 301 patients from a referral center.* Ann Oncol, 1997. **8**(7): p. 685-90.
76. Landry, C.S., et al., *Biochemical prognostic indicators for pancreatic neuroendocrine tumors and small bowel neuroendocrine tumors.* Gland Surg, 2014. **3**(4): p. 215-8.
77. Modlin, I.M., et al., *The clinical utility of a novel blood-based multi-transcriptome assay for the diagnosis of neuroendocrine tumors of the gastrointestinal tract.* Am J Gastroenterol, 2015. **110**(8): p. 1223-32.

78. Bergestuen, D.S., et al., *Plasma CCN2/connective tissue growth factor is associated with right ventricular dysfunction in patients with neuroendocrine tumors.* BMC Cancer, 2010. **10**: p. 6.
79. Schimmack, S., et al., *The clinical implications and biologic relevance of neurofilament expression in gastroenteropancreatic neuroendocrine neoplasms.* Cancer, 2012. **118**(10): p. 2763-75.
80. Liu, B., et al., *alpha-Internexin: a novel biomarker for pancreatic neuroendocrine tumor aggressiveness.* J Clin Endocrinol Metab, 2014. **99**(5): p. E786-95.
81. Fotouhi, O., et al., *Proteomics Suggests a Role for APC-Survivin in Response to Somatostatin Analog Treatment of Neuroendocrine Tumors.* J Clin Endocrinol Metab, 2016. **101**(10): p. 3616-3627.
82. Grabowski, P., et al., *Nuclear survivin is a powerful novel prognostic marker in gastroenteropancreatic neuroendocrine tumor disease.* Neuroendocrinology, 2005. **81**(1): p. 1-9.
83. Fujino, M., et al., *Expression of glucose transporter-1 is correlated with hypoxia-inducible factor 1alpha and malignant potential in pancreatic neuroendocrine tumors.* Oncol Lett, 2016. **12**(5): p. 3337-3343.
84. Binderup, T., et al., *Gene Expression of Glucose Transporter 1 (GLUT1), Hexokinase 1 and Hexokinase 2 in Gastroenteropancreatic Neuroendocrine Tumors: Correlation with F-18-fluorodeoxyglucose Positron Emission Tomography and Cellular Proliferation.* Diagnostics (Basel), 2013. **3**(4): p. 372-84.
85. McDonald, T.J., et al., *Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue.* Biochem Biophys Res Commun, 1979. **90**(1): p. 227-33.
86. Korse, C.M., et al., *Multicenter evaluation of a new progastrin-releasing peptide (ProGRP) immunoassay across Europe and China.* Clin Chim Acta, 2015. **438**: p. 388-95.
87. Holbourn, K.P., K.R. Acharya, and B. Perbal, *The CCN family of proteins: structure-function relationships.* Trends Biochem Sci, 2008. **33**(10): p. 461-73.
88. Schuller, M., D. Jenne, and R. Voltz, *The human PNMA family: novel neuronal proteins implicated in paraneoplastic neurological disease.* J Neuroimmunol, 2005. **169**(1-2): p. 172-6.
89. Nathke, I., *Cytoskeleton out of the cupboard: colon cancer and cytoskeletal changes induced by loss of APC.* Nat Rev Cancer, 2006. **6**(12): p. 967-74.
90. Mihaly, S.R., J. Ninomiya-Tsuji, and S. Morioka, *TAK1 control of cell death.* Cell Death Differ, 2014. **21**(11): p. 1667-76.
91. Obexer, P. and M.J. Ausserlechner, *X-linked inhibitor of apoptosis protein - a critical death resistance regulator and therapeutic target for personalized cancer therapy.* Front Oncol, 2014. **4**: p. 197.
92. Augeri, D.J., et al., *Inhibition of BMP and of TGFbeta receptors downregulates expression of XIAP and TAK1 leading to lung cancer cell death.* Mol Cancer, 2016. **15**: p. 27.
93. Cingarlini, S., et al., *Profiling mTOR pathway in neuroendocrine tumors.* Target Oncol, 2012. **7**(3): p. 183-8.
94. Yap, T.A., et al., *First-in-man clinical trial of the oral pan-AKT inhibitor MK-2206 in patients with advanced solid tumors.* J Clin Oncol, 2011. **29**(35): p. 4688-95.
95. Clavo, A.C., R.S. Brown, and R.L. Wahl, *Fluorodeoxyglucose uptake in human cancer cell lines is increased by hypoxia.* J Nucl Med, 1995. **36**(9): p. 1625-32.
96. Ozbudak, I.H., et al., *Glucose transporter-1 in pulmonary neuroendocrine carcinomas: expression and survival analysis.* Mod Pathol, 2009. **22**(5): p. 633-8.
97. Benzerdjeb, N., P. Berna, and H. Sevestre, *GLUT1: A novel tool reflecting proliferative activity of lung neuroendocrine tumors?* Pathol Int, 2017. **67**(1): p. 32-36.

98. Lin, W.W. and S.L. Hsieh, *Decoy receptor 3: a pleiotropic immunomodulator and biomarker for inflammatory diseases, autoimmune diseases and cancer.* Biochem Pharmacol, 2011. **81**(7): p. 838-47.
99. Casado, E., et al., *Upregulation of trefoil factor 3 (TFF3) after rectal cancer chemoradiotherapy is an adverse prognostic factor and a potential therapeutic target.* Int J Radiat Oncol Biol Phys, 2012. **84**(5): p. 1151-8.
100. Huang, Y.G., et al., *Aberrant expression of trefoil factor 3 is associated with colorectal carcinoma metastasis.* J Cancer Res Ther, 2013. **9**(3): p. 376-80.
101. Vestergaard, E.M., et al., *Promoter hypomethylation and upregulation of trefoil factors in prostate cancer.* Int J Cancer, 2010. **127**(8): p. 1857-65.
102. Jones, D.R., *Measuring midkine: the utility of midkine as a biomarker in cancer and other diseases.* Br J Pharmacol, 2014. **171**(12): p. 2925-39.
103. Hu, R., et al., *Increased drug efflux along with midkine gene high expression in childhood B-lineage acute lymphoblastic leukemia cells.* Int J Hematol, 2010. **92**(1): p. 105-10.
104. Wu, X., et al., *Midkine as a potential diagnostic marker in epithelial ovarian cancer for cisplatin/paclitaxel combination clinical therapy.* Am J Cancer Res, 2015. **5**(2): p. 629-38.
105. Heaphy, C.M., et al., *Altered telomeres in tumors with ATRX and DAXX mutations.* Science, 2011. **333**(6041): p. 425.
106. Schwartzentruber, J., et al., *Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma.* Nature, 2012. **482**(7384): p. 226-31.
107. Kulke, M.H., et al., *O6-methylguanine DNA methyltransferase deficiency and response to temozolomide-based therapy in patients with neuroendocrine tumors.* Clin Cancer Res, 2009. **15**(1): p. 338-45.
108. Schmitt, A.M., et al., *Prognostic and predictive roles of MGMT protein expression and promoter methylation in sporadic pancreatic neuroendocrine neoplasms.* Neuroendocrinology, 2014. **100**(1): p. 35-44.
109. Diaz, L.A., Jr. and A. Bardelli, *Liquid biopsies: genotyping circulating tumor DNA.* J Clin Oncol, 2014. **32**(6): p. 579-86.
110. Frederiksen, C.M., et al., *Classification of Dukes' B and C colorectal cancers using expression arrays.* J Cancer Res Clin Oncol, 2003. **129**(5): p. 263-71.
111. Hess, K.R., et al., *Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer.* J Clin Oncol, 2006. **24**(26): p. 4236-44.
112. van 't Veer, L.J., et al., *Gene expression profiling predicts clinical outcome of breast cancer.* Nature, 2002. **415**(6871): p. 530-6.
113. Modlin, I.M., et al., *Blood transcript analysis and metastatic recurrent small bowel carcinoid management.* BMC Cancer, 2014. **14**: p. 564.
114. Cwikla, J.B., et al., *Circulating Transcript Analysis (NETest) in GEP-NETs Treated With Somatostatin Analogs Defines Therapy.* J Clin Endocrinol Metab, 2015. **100**(11): p. E1437-45.
115. Di Leva, G. and C.M. Croce, *miRNA profiling of cancer.* Curr Opin Genet Dev, 2013. **23**(1): p. 3-11.
116. Arvidsson, Y., et al., *miRNA profiling of small intestinal neuroendocrine tumors defines novel molecular subtypes and identifies miR-375 as a biomarker of patient survival.* Mod Pathol, 2018.
117. Thorns, C., et al., *Global microRNA profiling of pancreatic neuroendocrine neoplasias.* Anticancer Res, 2014. **34**(5): p. 2249-54.

118. Vicentini, C., et al., *Clinical application of microRNA testing in neuroendocrine tumors of the gastrointestinal tract.* Molecules, 2014. **19**(2): p. 2458-68.
119. Oberg, K., et al., *A Delphic consensus assessment: imaging and biomarkers in gastroenteropancreatic neuroendocrine tumor disease management.* Endocr Connect, 2016. **5**(5): p. 174-87.
120. Volante, M., et al., *Somatostatin, cortistatin and their receptors in tumours.* Mol Cell Endocrinol, 2008. **286**(1-2): p. 219-29.
121. Korner, M., *Specific biology of neuroendocrine tumors: Peptide receptors as molecular targets.* Best Pract Res Clin Endocrinol Metab, 2016. **30**(1): p. 19-31.
122. de Herder, W.W., et al., *Somatostatin receptors in gastroentero-pancreatic neuroendocrine tumours.* Endocr Relat Cancer, 2003. **10**(4): p. 451-8.
123. Kaemmerer, D., et al., *Somatostatin receptors in bronchopulmonary neuroendocrine neoplasms: new diagnostic, prognostic, and therapeutic markers.* J Clin Endocrinol Metab, 2015. **100**(3): p. 831-40.
124. Volante, M., F. Bozzalla-Cassione, and M. Papotti, *Somatostatin receptors and their interest in diagnostic pathology.* Endocr Pathol, 2004. **15**(4): p. 275-91.
125. Duran-Prado, M., et al., *The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells.* Oncogene, 2012. **31**(16): p. 2049-61.
126. Duran-Prado, M., et al., *Identification and characterization of two novel truncated but functional isoforms of the somatostatin receptor subtype 5 differentially present in pituitary tumors.* J Clin Endocrinol Metab, 2009. **94**(7): p. 2634-43.
127. Durán-Prado, M., et al., *A potential inhibitory role for the new truncated variant of somatostatin receptor 5 (sst5TMD4) in pituitary adenomas poorly responsive to somatostatin analogs.* J Clin Endocrinol Metab, 2010. **95**(5): p. 2497-502.
128. Luque, R.M., et al., *Truncated somatostatin receptor variant sst5TMD4 confers aggressive features (proliferation, invasion and reduced octreotide response) to somatotropinomas.* Cancer Lett, 2015. **359**(2): p. 299-306.
129. Mole, D., et al., *The expression of the truncated isoform of somatostatin receptor subtype 5 associates with aggressiveness in medullary thyroid carcinoma cells.* Endocrine, 2015. **50**(2): p. 442-52.
130. Puig-Domingo, M., et al., *The truncated isoform of somatostatin receptor5 (sst5TMD4) is associated with poorly differentiated thyroid cancer.* PLoS One, 2014. **9**(1): p. e85527.
131. Sampedro-Nunez, M., et al., *Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors.* Oncotarget, 2015. **7**(6): p. 6593-608.
132. Sampedro-Nunez, M., et al., *Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors.* Oncotarget, 2016. **7**(6): p. 6593-608.
133. Pavel, M., et al., *ENETS Consensus Guidelines Update for the Management of Distant Metastatic Disease of Intestinal, Pancreatic, Bronchial Neuroendocrine Neoplasms (NEN) and NEN of Unknown Primary Site.* Neuroendocrinology, 2016. **103**(2): p. 172-85.
134. Falconi, M., et al., *ENETS Consensus Guidelines Update for the Management of Patients with Functional Pancreatic Neuroendocrine Tumors and Non-Functional Pancreatic Neuroendocrine Tumors.* Neuroendocrinology, 2016. **103**(2): p. 153-71.
135. Baldelli, R., et al., *Somatostatin analogs therapy in gastroenteropancreatic neuroendocrine tumors: current aspects and new perspectives.* Front Endocrinol (Lausanne), 2014. **5**: p. 7.

136. Delle Fave, G., et al., *ENETS Consensus Guidelines Update for Gastroduodenal Neuroendocrine Neoplasms.* Neuroendocrinology, 2016. **103**(2): p. 119-24.
137. Wangberg, B., et al., *[Somatostatin receptors. A new way to diagnosis and therapy of neuroendocrine tumors].* Lakartidningen, 1997. **94**(10): p. 829-30, 835-8.
138. Gahete, M.D., et al., *Ghrelin gene products, receptors, and GOAT enzyme: biological and pathophysiological insight.* J Endocrinol, 2014. **220**(1): p. R1-24.
139. Chopin, L.K., et al., *The ghrelin axis--does it have an appetite for cancer progression?* Endocr Rev, 2012. **33**(6): p. 849-91.
140. Kojima, M., et al., *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature, 1999. **402**(6762): p. 656-60.
141. Muller, T.D., et al., *Ghrelin.* Mol Metab, 2015. **4**(6): p. 437-60.
142. Nishi, Y., et al., *Structures and molecular forms of the ghrelin-family peptides.* Peptides, 2011. **32**(11): p. 2175-82.
143. Gahete, M.D., et al., *A novel human ghrelin variant (In1-ghrelin) and ghrelin-O-acyltransferase are overexpressed in breast cancer: potential pathophysiological relevance.* PLoS One, 2011. **6**(8): p. e23302.
144. Leung, P.K., et al., *The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor.* Cell Signal, 2007. **19**(5): p. 1011-22.
145. Hormaechea-Agulla, D., et al., *Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer, and its levels are associated with patient's metabolic status: Potential value as a non-invasive biomarker.* Cancer Lett, 2016. **383**(1): p. 125-134.
146. Pokuri, V.K., M.K. Fong, and R. Iyer, *Octreotide and Lanreotide in Gastroenteropancreatic Neuroendocrine Tumors.* Curr Oncol Rep, 2016. **18**(1): p. 7.
147. Ucan, B., et al., *Serum ghrelin levels in papillary thyroid carcinoma.* Arch Endocrinol Metab, 2017. **61**(5): p. 464-469.
148. Ibanez-Costa, A., et al., *In1-ghrelin splicing variant is overexpressed in pituitary adenomas and increases their aggressive features.* Sci Rep, 2015. **5**: p. 8714.
149. Luque, R.M., et al., *In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters.* Oncotarget, 2015. **6**(23): p. 19619-33.
150. Kanakis, G., et al., *Expression of Somatostatin Receptors 1-5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role.* Neuroendocrinology, 2015. **101**(3): p. 211-22.
151. Corbetta, S., et al., *Circulating ghrelin levels in patients with pancreatic and gastrointestinal neuroendocrine tumors: identification of one pancreatic ghrelinoma.* J Clin Endocrinol Metab, 2003. **88**(7): p. 3117-20.
152. Gilbert, J.A., et al., *Molecular markers for novel therapies in neuroendocrine (carcinoid) tumors.* Endocr Relat Cancer, 2010. **17**(3): p. 623-36.
153. Rickman, O.B., et al., *Analysis of ErbB receptors in pulmonary carcinoid tumors.* Clin Cancer Res, 2009. **15**(10): p. 3315-24.
154. Shah, T., et al., *Epidermal growth factor receptor expression and activation in neuroendocrine tumours.* J Neuroendocrinol, 2006. **18**(5): p. 355-60.
155. Srivastava, A., et al., *Immunohistochemical expression of transforming growth factor alpha and epidermal growth factor receptor in pancreatic endocrine tumors.* Hum Pathol, 2001. **32**(11): p. 1184-9.
156. Pavel, M.E., et al., *Circulating levels of angiogenic cytokines can predict tumour progression and prognosis in neuroendocrine carcinomas.* Clin Endocrinol (Oxf), 2005. **62**(4): p. 434-43.

157. Zurita, A.J., et al., *Circulating cytokines and monocyte subpopulations as biomarkers of outcome and biological activity in sunitinib-treated patients with advanced neuroendocrine tumours.* Br J Cancer, 2015. **112**(7): p. 1199-205.
158. Khan, M.S., et al., *Circulating tumor cells as prognostic markers in neuroendocrine tumors.* J Clin Oncol, 2013. **31**(3): p. 365-72.
159. Khan, M.S., et al., *Circulating tumor cells and EpCAM expression in neuroendocrine tumors.* Clin Cancer Res, 2011. **17**(2): p. 337-45.
160. Hilfenhaus, G., et al., *Placental growth factor supports neuroendocrine tumor growth and predicts disease prognosis in patients.* Endocr Relat Cancer, 2013. **20**(3): p. 305-19.
161. Iyer, G., et al., *Genome sequencing identifies a basis for everolimus sensitivity.* Science, 2012. **338**(6104): p. 221.
162. Ramage, J.K., et al., *ENETS Consensus Guidelines Update for Colorectal Neuroendocrine Neoplasms.* Neuroendocrinology, 2016. **103**(2): p. 139-43.
163. Maxwell, J.E. and J.R. Howe, *Imaging in neuroendocrine tumors: an update for the clinician.* Int J Endocr Oncol, 2015. **2**(2): p. 159-168.
164. de Herder, W.W., et al., *Somatostatin receptor imaging for neuroendocrine tumors.* Pituitary, 2006. **9**(3): p. 243-8.
165. Kwekkeboom, D.J., et al., *Somatostatin-receptor-based imaging and therapy of gastroenteropancreatic neuroendocrine tumors.* Endocr Relat Cancer, 2010. **17**(1): p. R53-73.
166. Panagiotidis, E., et al., *Comparison of the Impact of 68Ga-DOTATATE and 18F-FDG PET/CT on Clinical Management in Patients with Neuroendocrine Tumors.* J Nucl Med, 2017. **58**(1): p. 91-96.
167. Van Binnebeek, S., et al., *Comparison of diagnostic accuracy of (111)In-pentetreotide SPECT and (68)Ga-DOTATOC PET/CT: A lesion-by-lesion analysis in patients with metastatic neuroendocrine tumours.* Eur Radiol, 2016. **26**(3): p. 900-9.
168. Kayani, I., et al., *A comparison of 68Ga-DOTATATE and 18F-FDG PET/CT in pulmonary neuroendocrine tumors.* J Nucl Med, 2009. **50**(12): p. 1927-32.
169. Sowa-Staszczak, A., et al., *99mTc Labeled Glucagon-Like Peptide-1-Analogue (99mTc-GLP1) Scintigraphy in the Management of Patients with Occult Insulinoma.* PLoS One, 2016. **11**(8): p. e0160714.
170. Raphael, M.J., et al., *Principles of diagnosis and management of neuroendocrine tumours.* CMAJ, 2017. **189**(10): p. E398-E404.
171. de Herder, W.W., et al., *Considerations concerning a tailored, individualized therapeutic management of patients with (neuro)endocrine tumours of the gastrointestinal tract and pancreas.* Endocr Relat Cancer, 2004. **11**(1): p. 19-34.
172. Akerstrom, G., et al., *Management of midgut carcinoids.* J Surg Oncol, 2005. **89**(3): p. 161-9.
173. Modlin, I.M., et al., *Gastroenteropancreatic neuroendocrine tumours.* Lancet Oncol, 2008. **9**(1): p. 61-72.
174. Reincke, M., et al., *A critical reappraisal of bilateral adrenalectomy for ACTH-dependent Cushing's syndrome.* Eur J Endocrinol, 2015. **173**(4): p. M23-32.
175. Garcia-Carbonero, R., et al., *ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Neoplasms. Systemic Therapy 2: Chemotherapy.* Neuroendocrinology, 2017. **105**(3): p. 281-294.
176. Davis, G.R., et al., *Effect of somatostatin infusion on jejunal water and electrolyte transport in a patient with secretory diarrhea due to malignant carcinoid syndrome.* Gastroenterology, 1980. **78**(2): p. 346-9.

177. Dharmsathaphorn, K., et al., *Somatostatin inhibits diarrhea in the carcinoid syndrome.* Ann Intern Med, 1980. **92**(1): p. 68-9.
178. Rubin, J., et al., *Octreotide acetate long-acting formulation versus open-label subcutaneous octreotide acetate in malignant carcinoid syndrome.* J Clin Oncol, 1999. **17**(2): p. 600-6.
179. Kvols, L.K., et al., *Treatment of the malignant carcinoid syndrome. Evaluation of a long-acting somatostatin analogue.* N Engl J Med, 1986. **315**(11): p. 663-6.
180. O'Toole, D., et al., *Treatment of carcinoid syndrome: a prospective crossover evaluation of lanreotide versus octreotide in terms of efficacy, patient acceptability, and tolerance.* Cancer, 2000. **88**(4): p. 770-6.
181. Ruszniewski, P., et al., *Rapid and sustained relief from the symptoms of carcinoid syndrome: results from an open 6-month study of the 28-day prolonged-release formulation of lanreotide.* Neuroendocrinology, 2004. **80**(4): p. 244-51.
182. Teunissen, J.J., D.J. Kwekkeboom, and E.P. Krenning, *Quality of life in patients with gastroenteropancreatic tumors treated with [177Lu-DOTA0,Tyr3]octreotate.* J Clin Oncol, 2004. **22**(13): p. 2724-9.
183. Waldherr, C., et al., *Tumor response and clinical benefit in neuroendocrine tumors after 7.4 GBq (90)Y-DOTATOC.* J Nucl Med, 2002. **43**(5): p. 610-6.
184. Kvols, L.K., et al., *Pasireotide (SOM230) shows efficacy and tolerability in the treatment of patients with advanced neuroendocrine tumors refractory or resistant to octreotide LAR: results from a phase II study.* Endocr Relat Cancer, 2012. **19**(5): p. 657-66.
185. Frank, M., et al., *Combination therapy with octreotide and alpha-interferon: effect on tumor growth in metastatic endocrine gastroenteropancreatic tumors.* Am J Gastroenterol, 1999. **94**(5): p. 1381-7.
186. Pavel, M., et al., *Telotristat ethyl in carcinoid syndrome: safety and efficacy in the TELECAST phase 3 trial.* Endocr Relat Cancer, 2018. **25**(3): p. 309-322.
187. Anderson, A.S., D. Krauss, and R. Lang, *Cardiovascular complications of malignant carcinoid disease.* Am Heart J, 1997. **134**(4): p. 693-702.
188. Modlin, I.M., et al., *Therapeutic options for gastrointestinal carcinoids.* Clin Gastroenterol Hepatol, 2006. **4**(5): p. 526-47.
189. Moller, J.E., et al., *Factors associated with progression of carcinoid heart disease.* N Engl J Med, 2003. **348**(11): p. 1005-15.
190. Kamp, K., et al., *Parathyroid hormone-related peptide (PTHrP) secretion by gastroenteropancreatic neuroendocrine tumors (GEP-NETs): clinical features, diagnosis, management, and follow-up.* J Clin Endocrinol Metab, 2014. **99**(9): p. 3060-9.
191. Ilias, I., et al., *Cushing's syndrome due to ectopic corticotropin secretion: twenty years' experience at the National Institutes of Health.* J Clin Endocrinol Metab, 2005. **90**(8): p. 4955-62.
192. Kamp, K., et al., *Prevalence and clinical features of the ectopic ACTH syndrome in patients with gastroenteropancreatic and thoracic neuroendocrine tumors.* Eur J Endocrinol, 2016. **174**(3): p. 271-80.
193. van der Pas, R., et al., *New developments in the medical treatment of Cushing's syndrome.* Endocr Relat Cancer, 2012. **19**(6): p. R205-23.
194. Aniszewski, J.P., et al., *Cushing syndrome due to ectopic adrenocorticotropic hormone secretion.* World J Surg, 2001. **25**(7): p. 934-40.
195. Lamberts, S.W., et al., *A role of (labeled) somatostatin analogs in the differential diagnosis and treatment of Cushing's syndrome.* J Clin Endocrinol Metab, 1994. **78**(1): p. 17-9.

196. Phlipponneau, M., et al., *Somatostatin analogs for the localization and preoperative treatment of an adrenocorticotropin-secreting bronchial carcinoid tumor.* J Clin Endocrinol Metab, 1994. **78**(1): p. 20-4.
197. Pivonello, R., et al., *Dopamine receptor expression and function in corticotroph ectopic tumors.* J Clin Endocrinol Metab, 2007. **92**(1): p. 65-9.
198. Pivonello, R., et al., *Cabergoline plus lanreotide for ectopic Cushing's syndrome.* N Engl J Med, 2005. **352**(23): p. 2457-8.
199. Krenning, E.P., et al., *Somatostatin receptor scintigraphy in carcinoids, gastrinomas and Cushing's syndrome.* Digestion, 1994. **55 Suppl 3**: p. 54-9.
200. de Herder, W.W., et al., *Somatostatin receptor scintigraphy: its value in tumor localization in patients with Cushing's syndrome caused by ectopic corticotropin or corticotropin-releasing hormone secretion.* Am J Med, 1994. **96**(4): p. 305-12.
201. Baudry, C., A.C. Paepegaey, and L. Groussin, *Reversal of Cushing's syndrome by vandetanib in medullary thyroid carcinoma.* N Engl J Med, 2013. **369**(6): p. 584-6.
202. Barroso-Sousa, R., et al., *Complete resolution of hypercortisolism with sorafenib in a patient with advanced medullary thyroid carcinoma and ectopic ACTH (adrenocorticotropic hormone) syndrome.* Thyroid, 2014. **24**(6): p. 1062-6.
203. Kamenicky, P., et al., *Mitotane, metyrapone, and ketoconazole combination therapy as an alternative to rescue adrenalectomy for severe ACTH-dependent Cushing's syndrome.* J Clin Endocrinol Metab, 2011. **96**(9): p. 2796-804.
204. Schulte, H.M., et al., *Infusion of low dose etomidate: correction of hypercortisolemia in patients with Cushing's syndrome and dose-response relationship in normal subjects.* J Clin Endocrinol Metab, 1990. **70**(5): p. 1426-30.
205. Nieman, L.K., et al., *Treatment of Cushing's Syndrome: An Endocrine Society Clinical Practice Guideline.* J Clin Endocrinol Metab, 2015. **100**(8): p. 2807-31.
206. Goode, P.N., et al., *Diazoxide in the management of patients with insulinoma.* World J Surg, 1986. **10**(4): p. 586-92.
207. Gill, G.V., O. Rauf, and I.A. MacFarlane, *Diazoxide treatment for insulinoma: a national UK survey.* Postgrad Med J, 1997. **73**(864): p. 640-1.
208. de Herder, W.W., et al., *New therapeutic options for metastatic malignant insulinomas.* Clin Endocrinol (Oxf), 2011. **75**(3): p. 277-84.
209. Baudin, E., et al., *Malignant insulinoma: recommendations for characterisation and treatment.* Ann Endocrinol (Paris), 2013. **74**(5-6): p. 523-33.
210. Altszuler, N., E. Moraru, and J. Hampshire, *On the mechanism of diazoxide-induced hyperglycemia.* Diabetes, 1977. **26**(10): p. 931-5.
211. Singh, V., et al., *Characterization of somatostatin receptor subtype-specific regulation of insulin and glucagon secretion: an in vitro study on isolated human pancreatic islets.* J Clin Endocrinol Metab, 2007. **92**(2): p. 673-80.
212. Fuhrer, D.K., M. Kobayashi, and H. Jiang, *Insulin release and suppression by tacrolimus, rapamycin and cyclosporin A are through regulation of the ATP-sensitive potassium channel.* Diabetes Obes Metab, 2001. **3**(6): p. 393-402.
213. Di Paolo, S., et al., *Chronic inhibition of mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2 and AKT activation: A crossroad between cancer and diabetes?* J Am Soc Nephrol, 2006. **17**(8): p. 2236-44.
214. Fiebrich, H.B., et al., *Everolimus induces rapid plasma glucose normalization in insulinoma patients by effects on tumor as well as normal tissues.* Oncologist, 2011. **16**(6): p. 783-7.

215. Kulke, M.H., E.K. Bergsland, and J.C. Yao, *Glycemic control in patients with insulinoma treated with everolimus.* N Engl J Med, 2009. **360**(2): p. 195-7.
216. Santangelo, W.C., et al., *Somatostatin analog-induced remission of necrolytic migratory erythema without changes in plasma glucagon concentration.* Pancreas, 1986. **1**(5): p. 464-9.
217. Schmid, H.A., *Pasireotide (SOM230): development, mechanism of action and potential applications.* Mol Cell Endocrinol, 2008. **286**(1-2): p. 69-74.
218. Raymond, E., et al., *Sunitinib malate for the treatment of pancreatic neuroendocrine tumors.* N Engl J Med, 2011. **364**(6): p. 501-13.
219. Yao, J.C., et al., *Everolimus for advanced pancreatic neuroendocrine tumors.* N Engl J Med, 2011. **364**(6): p. 514-23.
220. Kwekkeboom, D.J., et al., *Treatment with the radiolabeled somatostatin analog [177 Lu-DOTA 0,Tyr3]octreotate: toxicity, efficacy, and survival.* J Clin Oncol, 2008. **26**(13): p. 2124-30.
221. Peghini, P.L., et al., *Effect of chronic hypergastrinemia on human enterochromaffin-like cells: insights from patients with sporadic gastrinomas.* Gastroenterology, 2002. **123**(1): p. 68-85.
222. Eriksson, B., et al., *High-dose treatment with lanreotide of patients with advanced neuroendocrine gastrointestinal tumors: clinical and biological effects.* Ann Oncol, 1997. **8**(10): p. 1041-4.
223. O'Dorisio, T.M., H.S. Mekhjian, and T.S. Gaginella, *Medical therapy of VIPomas.* Endocrinol Metab Clin North Am, 1989. **18**(2): p. 545-56.
224. Delbaldo, C., et al., *Sunitinib in advanced pancreatic neuroendocrine tumors: latest evidence and clinical potential.* Ther Adv Med Oncol, 2012. **4**(1): p. 9-18.
225. Faivre, S., et al., *Sunitinib in Pancreatic Neuroendocrine Tumors: Updated Progression-Free Survival and Final Overall Survival From a Phase III Randomized Study.* Ann Oncol, 2016.
226. van der Zwan, W.A., et al., *GEPNETs update: Radionuclide therapy in neuroendocrine tumors.* Eur J Endocrinol, 2015. **172**(1): p. R1-8.
227. Giustina, A., et al., *Diagnosis and treatment of acromegaly complications.* J Endocrinol Invest, 2003. **26**(12): p. 1242-7.
228. van Hoek, M., et al., *Effects of somatostatin analogs on a growth hormone-releasing hormone secreting bronchial carcinoid, in vivo and in vitro studies.* J Clin Endocrinol Metab, 2009. **94**(2): p. 428-33.
229. Green, J.R., *Bisphosphonates: preclinical review.* Oncologist, 2004. **9 Suppl 4**: p. 3-13.
230. Loftus, L.S., S. Edwards-Bennett, and G.H. Sokol, *Systemic therapy for bone metastases.* Cancer Control, 2012. **19**(2): p. 145-53.
231. Oberg, K., et al., *Consensus report on the use of somatostatin analogs for the management of neuroendocrine tumors of the gastroenteropancreatic system.* Ann Oncol, 2004. **15**(6): p. 966-73.
232. Jensen, R.T., *Carcinoid and pancreatic endocrine tumors: recent advances in molecular pathogenesis, localization, and treatment.* Curr Opin Oncol, 2000. **12**(4): p. 368-77.
233. Veenstra, M.J., et al., *Targeting the somatostatin receptor in pituitary and neuroendocrine tumors.* Expert Opin Ther Targets, 2013. **17**(11): p. 1329-43.
234. Rinke, A., et al., *Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID Study Group.* J Clin Oncol, 2009. **27**(28): p. 4656-63.
235. Rinke, A., et al., *Placebo-Controlled, Double-Blind, Prospective, Randomized Study on the Effect of Octreotide LAR in the Control of Tumor Growth in Patients with Metastatic Neuroendocrine Midgut Tumors (PROMID): Results of Long-Term Survival.* Neuroendocrinology, 2017. **104**(1): p. 26-32.

236. Caplin, M.E., M. Pavel, and P. Ruszniewski, *Lanreotide in metastatic enteropancreatic neuroendocrine tumors.* N Engl J Med, 2014. **371**(16): p. 1556-7.
237. Zomerhuis, M.T., et al., *Octreotide exerts only acute, but no sustained, effects on MRI enhancement of liver metastases in carcinoid syndrome.* Neuroendocrinology, 2005. **82**(1): p. 41-8.
238. Yao, J.C., et al., *Phase I dose-escalation study of long-acting pasireotide in patients with neuroendocrine tumors.* Onco Targets Ther, 2017. **10**: p. 3177-3186.
239. Cives, M., et al., *Phase II clinical trial of pasireotide long-acting repeatable in patients with metastatic neuroendocrine tumors.* Endocr Relat Cancer, 2015. **22**(1): p. 1-9.
240. Wolin, E.M., et al., *Phase III study of pasireotide long-acting release in patients with metastatic neuroendocrine tumors and carcinoid symptoms refractory to available somatostatin analogues.* Drug Des Devel Ther, 2015. **9**: p. 5075-86.
241. Ferolla, P., et al., *Efficacy and safety of long-acting pasireotide or everolimus alone or in combination in patients with advanced carcinoids of the lung and thymus (LUNA): an open-label, multicentre, randomised, phase 2 trial.* Lancet Oncol, 2017. **18**(12): p. 1652-1664.
242. Kulke, M.H., et al., *A randomized, open-label, phase 2 study of everolimus in combination with pasireotide LAR or everolimus alone in advanced, well-differentiated, progressive pancreatic neuroendocrine tumors: COOPERATE-2 trial.* Ann Oncol, 2017. **28**(6): p. 1309-1315.
243. Oberg, K. and B. Eriksson, *The role of interferons in the management of carcinoid tumours.* Br J Haematol, 1991. **79 Suppl 1**: p. 74-7.
244. Oberg, K., K. Funa, and G. Alm, *Effects of leukocyte interferon on clinical symptoms and hormone levels in patients with mid-gut carcinoid tumors and carcinoid syndrome.* N Engl J Med, 1983. **309**(3): p. 129-33.
245. Plockinger, U., et al., *Guidelines for the diagnosis and treatment of neuroendocrine gastrointestinal tumours. A consensus statement on behalf of the European Neuroendocrine Tumour Society (ENETS).* Neuroendocrinology, 2004. **80**(6): p. 394-424.
246. Oberg, K., *Interferon-alpha versus somatostatin or the combination of both in gastro-enteropancreatic tumours.* Digestion, 1996. **57 Suppl 1**: p. 81-3.
247. Yao, J.C., et al., *Phase III Prospective Randomized Comparison Trial of Depot Octreotide Plus Interferon Alfa-2b Versus Depot Octreotide Plus Bevacizumab in Patients With Advanced Carcinoid Tumors: SWOG S0518.* J Clin Oncol, 2017. **35**(15): p. 1695-1703.
248. Pavel, M.E., et al., *Efficacy and tolerability of pegylated IFN-alpha in patients with neuroendocrine gastroenteropancreatic carcinomas.* J Interferon Cytokine Res, 2006. **26**(1): p. 8-13.
249. Zandee, W.T. and W.W. de Herder, *The Evolution of Neuroendocrine Tumor Treatment Reflected by ENETS Guidelines.* Neuroendocrinology, 2018. **106**(4): p. 357-365.
250. Kwekkeboom, D.J., et al., *Radiolabeled somatostatin analog [177Lu-DOTA0,Tyr3]octreotate in patients with endocrine gastroenteropancreatic tumors.* J Clin Oncol, 2005. **23**(12): p. 2754-62.
251. Brabander, T., et al., *Long-Term Efficacy, Survival, and Safety of [(177)Lu-DOTA(0),Tyr(3)] octreotate in Patients with Gastroenteropancreatic and Bronchial Neuroendocrine Tumors.* Clin Cancer Res, 2017. **23**(16): p. 4617-4624.
252. Ezziddin, S., et al., *Predictors of long-term outcome in patients with well-differentiated gastroenteropancreatic neuroendocrine tumors after peptide receptor radionuclide therapy with 177Lu-octreotate.* J Nucl Med, 2014. **55**(2): p. 183-90.
253. Strosberg, J., et al., *Phase 3 Trial of (177)Lu-Dotatate for Midgut Neuroendocrine Tumors.* N Engl J Med, 2017. **376**(2): p. 125-135.

254. Severi, S., et al., *Feasibility and utility of re-treatment with (177)Lu-DOTATATE in GEP-NENs relapsed after treatment with (90)Y-DOTATOC.* Eur J Nucl Med Mol Imaging, 2015. **42**(13): p. 1955-63.
255. Forrer, F., et al., *Treatment with 177Lu-DOTATOC of patients with relapse of neuroendocrine tumors after treatment with 90Y-DOTATOC.* J Nucl Med, 2005. **46**(8): p. 1310-6.
256. Claringbold, P.G., et al., *Phase II study of radiopeptide 177Lu-octreotate and capecitabine therapy of progressive disseminated neuroendocrine tumours.* Eur J Nucl Med Mol Imaging, 2011. **38**(2): p. 302-11.
257. Claringbold, P.G., R.A. Price, and J.H. Turner, *Phase I-II study of radiopeptide 177Lu-octreotate in combination with capecitabine and temozolomide in advanced low-grade neuroendocrine tumors.* Cancer Biother Radiopharm, 2012. **27**(9): p. 561-9.
258. Kong, G., et al., *Assessment of predictors of response and long-term survival of patients with neuroendocrine tumour treated with peptide receptor chemoradionuclide therapy (PRCRT).* Eur J Nucl Med Mol Imaging, 2014. **41**(10): p. 1831-44.
259. Claringbold, P.G. and J.H. Turner, *NeuroEndocrine Tumor Therapy with Lutetium-177-octreotate and Everolimus (NETTLE): A Phase I Study.* Cancer Biother Radiopharm, 2015. **30**(6): p. 261-9.
260. Barber, T.W., et al., *The potential for induction peptide receptor chemoradionuclide therapy to render inoperable pancreatic and duodenal neuroendocrine tumours resectable.* Eur J Surg Oncol, 2012. **38**(1): p. 64-71.
261. Ezziddin, S., et al., *Neoadjuvant downsizing by internal radiation: a case for preoperative peptide receptor radionuclide therapy in patients with pancreatic neuroendocrine tumors.* Clin Nucl Med, 2012. **37**(1): p. 102-4.
262. van Vliet, E.I., et al., *Neoadjuvant Treatment of Nonfunctioning Pancreatic Neuroendocrine Tumors with [177Lu-DOTA0,Tyr3]Octreotate.* J Nucl Med, 2015. **56**(11): p. 1647-53.
263. Chan, J. and M. Kulke, *Targeting the mTOR signaling pathway in neuroendocrine tumors.* Curr Treat Options Oncol, 2014. **15**(3): p. 365-79.
264. Yao, J.C., et al., *Everolimus for the Treatment of Advanced Pancreatic Neuroendocrine Tumors: Overall Survival and Circulating Biomarkers From the Randomized, Phase III RADIANT-3 Study.* J Clin Oncol, 2016. **34**(32): p. 3906-3913.
265. Lombard-Bohas, C., et al., *Impact of prior chemotherapy use on the efficacy of everolimus in patients with advanced pancreatic neuroendocrine tumors: a subgroup analysis of the phase III RADIANT-3 trial.* Pancreas, 2015. **44**(2): p. 181-9.
266. Anthony, L.B., et al., *Impact of Previous Somatostatin Analogue Use on the Activity of Everolimus in Patients with Advanced Neuroendocrine Tumors: Analysis from the Phase III RADIANT-2 Trial.* Neuroendocrinology, 2015. **102**(1-2): p. 18-25.
267. Yao, J.C., et al., *Everolimus for the treatment of advanced, non-functional neuroendocrine tumours of the lung or gastrointestinal tract (RADIANT-4): a randomised, placebo-controlled, phase 3 study.* Lancet, 2016. **387**(10022): p. 968-977.
268. Fazio, N., et al., *Everolimus plus octreotide long-acting repeatable in patients with advanced lung neuroendocrine tumors: analysis of the phase 3, randomized, placebo-controlled RADIANT-2 study.* Chest, 2013. **143**(4): p. 955-962.
269. Castellano, D., et al., *Everolimus plus octreotide long-acting repeatable in patients with colorectal neuroendocrine tumors: a subgroup analysis of the phase III RADIANT-2 study.* Oncologist, 2013. **18**(1): p. 46-53.

270. Wiedmann, M.W. and J. Mossner, *Safety and efficacy of sunitinib in patients with unresectable pancreatic neuroendocrine tumors.* Clin Med Insights Oncol, 2012. **6**: p. 381-93.
271. Aristizabal Prada, E.T. and C.J. Auernhammer, *Targeted therapy of gastroenteropancreatic neuroendocrine tumours: preclinical strategies and future targets.* Endocr Connect, 2018. **7**(1): p. R1-R25.
272. Pennock, G.K. and L.Q. Chow, *The Evolving Role of Immune Checkpoint Inhibitors in Cancer Treatment.* Oncologist, 2015. **20**(7): p. 812-22.
273. Chauhan, A., et al., *Immune checkpoint inhibitors in neuroendocrine tumors: A single institution experience with review of literature.* Oncotarget, 2018. **9**(10): p. 8801-8809.
274. Kim, S.T., et al., *The Impact of PD-L1 Expression in Patients with Metastatic GEP-NETs.* J Cancer, 2016. **7**(5): p. 484-9.
275. Herbst, R.S., et al., *Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients.* Nature, 2014. **515**(7528): p. 563-7.
276. Tsuruoka, K., et al., *PD-L1 expression in neuroendocrine tumors of the lung.* Lung Cancer, 2017. **108**: p. 115-120.
277. Fan, Y., et al., *Prognostic value of PD-L1 and PD-1 expression in pulmonary neuroendocrine tumors.* Onco Targets Ther, 2016. **9**: p. 6075-6082.
278. Cavalcanti, E., et al., *Role of PD-L1 expression as a biomarker for GEP neuroendocrine neoplasm grading.* Cell Death Dis, 2017. **8**(8): p. e3004.
279. Culler, M.D., *Somatostatin-dopamine chimeras: a novel approach to treatment of neuroendocrine tumors.* Horm Metab Res, 2011. **43**(12): p. 854-7.
280. Rocheville, M., et al., *Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers.* J Biol Chem, 2000. **275**(11): p. 7862-9.
281. Rocheville, M., et al., *Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity.* Science, 2000. **288**(5463): p. 154-7.
282. Couvelard, A., et al., *Antisecretory Effects of Chimeric Somatostatin/Dopamine Receptor Ligands on Gastroenteropancreatic Neuroendocrine Tumors.* Pancreas, 2017. **46**(5): p. 631-638.
283. Feelders, R.A. and L.J. Hofland, *Medical treatment of Cushing's disease.* J Clin Endocrinol Metab, 2013. **98**(2): p. 425-38.
284. Beardwell, C.G., A.R. Adamson, and S.M. Shalet, *Prolonged remission in florid Cushing's syndrome following metyrapone treatment.* Clin Endocrinol (Oxf), 1981. **14**(5): p. 485-92.
285. Loh, K.C., R. Gupta, and A.H. Shlossberg, *Spontaneous remission of ectopic Cushing's syndrome due to pheochromocytoma: a case report.* Eur J Endocrinol, 1996. **135**(4): p. 440-3.
286. Sharma, S.T. and L.K. Nieman, *Prolonged remission after long-term treatment with steroidogenesis inhibitors in Cushing's syndrome caused by ectopic ACTH secretion.* Eur J Endocrinol, 2012. **166**(3): p. 531-6.
287. Steen, R.E., et al., *In vivo and in vitro inhibition by ketoconazole of ACTH secretion from a human thymic carcinoid tumour.* Acta Endocrinol (Copenh), 1991. **125**(3): p. 331-4.
288. Rochlitz, C.F., et al., *Cytotoxicity of ketoconazole in malignant cell lines.* Cancer Chemother Pharmacol, 1988. **21**(4): p. 319-22.
289. Ho, Y.S., et al., *Ketoconazole-induced apoptosis through P53-dependent pathway in human colorectal and hepatocellular carcinoma cell lines.* Toxicol Appl Pharmacol, 1998. **153**(1): p. 39-47.
290. Forgue-Lafitte, M.E., et al., *Effects of ketoconazole on the proliferation and cell cycle of human cancer cell lines.* Cancer Res, 1992. **52**(24): p. 6827-31.
291. Evans, J.M., et al., *Metformin and reduced risk of cancer in diabetic patients.* BMJ, 2005. **330**(7503): p. 1304-5.

292. Li, D., et al., *Antidiabetic therapies affect risk of pancreatic cancer.* Gastroenterology, 2009. **137**(2): p. 482-8.
293. Lee, M.S., et al., *Type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in Taiwanese: a representative population prospective cohort study of 800,000 individuals.* BMC Cancer, 2011. **11**: p. 20.
294. Mazzone, P.J., et al., *The effect of metformin and thiazolidinedione use on lung cancer in diabetics.* BMC Cancer, 2012. **12**: p. 410.
295. Col, N.F., et al., *Metformin and breast cancer risk: a meta-analysis and critical literature review.* Breast Cancer Res Treat, 2012. **135**(3): p. 639-46.
296. Ma, S., et al., *Meta-analysis of studies using metformin as a reducer for liver cancer risk in diabetic patients.* Medicine (Baltimore), 2017. **96**(19): p. e6888.
297. Li, X., et al., *The effect of metformin on survival of patients with pancreatic cancer: a meta-analysis.* Sci Rep, 2017. **7**(1): p. 5825.
298. Vlotides, G., et al., *Anticancer effects of metformin on neuroendocrine tumor cells in vitro.* Hormones (Athens), 2014. **13**(4): p. 498-508.
299. Soares, H.P., et al., *Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells.* PLoS One, 2013. **8**(2): p. e57289.
300. Zhou, G., et al., *Role of AMP-activated protein kinase in mechanism of metformin action.* J Clin Invest, 2001. **108**(8): p. 1167-74.
301. Hawley, S.A., et al., *Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation.* Cell Metab, 2010. **11**(6): p. 554-65.
302. Micic, D., et al., *Metformin: its emerging role in oncology.* Hormones (Athens), 2011. **10**(1): p. 5-15.
303. Jalving, M., et al., *Metformin: taking away the candy for cancer?* Eur J Cancer, 2010. **46**(13): p. 2369-80.
304. Emami Riedmaier, A., et al., *Metformin and cancer: from the old medicine cabinet to pharmacological pitfalls and prospects.* Trends Pharmacol Sci, 2013. **34**(2): p. 126-35.
305. Dowling, R.J., et al., *Metformin in cancer: translational challenges.* J Mol Endocrinol, 2012. **48**(3): p. R31-43.
306. Luengo, A., L.B. Sullivan, and M.G. Heiden, *Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy.* BMC Biol, 2014. **12**: p. 82.
307. Gazzerro, P., et al., *Pharmacological actions of statins: a critical appraisal in the management of cancer.* Pharmacol Rev, 2012. **64**(1): p. 102-46.
308. Maj, M., et al., *Anti-proliferative and cytotoxic activity of rosuvastatin against melanoma cells.* Postepy Dermatol Alergol, 2016. **33**(4): p. 257-62.
309. Jang, H.J., et al., *Statin induces apoptosis of human colon cancer cells and downregulation of insulin-like growth factor 1 receptor via proapoptotic ERK activation.* Oncol Lett, 2016. **12**(1): p. 250-256.
310. Bjarnadottir, O., et al., *Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial.* Breast Cancer Res Treat, 2013. **138**(2): p. 499-508.
311. Garwood, E.R., et al., *Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer.* Breast Cancer Res Treat, 2010. **119**(1): p. 137-44.
312. Brozovic, A., et al., *Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance.* Int J Cancer, 2004. **112**(6): p. 974-85.

313. Koyuturk, M., M. Ersoz, and N. Altiok, *Simvastatin induces apoptosis in human breast cancer cells: p53 and estrogen receptor independent pathway requiring signalling through JNK.* Cancer Lett, 2007. **250**(2): p. 220-8.
314. Denoyelle, C., et al., *Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study.* Carcinogenesis, 2001. **22**(8): p. 1139-48.
315. Mazzaglia, P.J., et al., *Laparoscopic radiofrequency ablation of neuroendocrine liver metastases: a 10-year experience evaluating predictors of survival.* Surgery, 2007. **142**(1): p. 10-9.
316. Mayo, S.C., et al., *Surgical management of hepatic neuroendocrine tumor metastasis: results from an international multi-institutional analysis.* Ann Surg Oncol, 2010. **17**(12): p. 3129-36.
317. Vogl, T.J., et al., *Liver metastases of neuroendocrine carcinomas: interventional treatment via transarterial embolization, chemoembolization and thermal ablation.* Eur J Radiol, 2009. **72**(3): p. 517-28.
318. Dong, X.D. and B.I. Carr, *Hepatic artery chemoembolization for the treatment of liver metastases from neuroendocrine tumors: a long-term follow-up in 123 patients.* Med Oncol, 2011. **28 Suppl 1**: p. S286-90.
319. Kennedy, A., et al., *Role of hepatic intra-arterial therapies in metastatic neuroendocrine tumours (NET): guidelines from the NET-Liver-Metastases Consensus Conference.* HPB (Oxford), 2015. **17**(1): p. 29-37.

PART I

Novel diagnostic markers for neuroendocrine tumors

Chapter 2

The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features

Herrera-Martínez AD[1,2], Gahete MD[1,3], Sánchez-Sánchez R[1,2], Salas RO[1,2], Serrano-Blanch R[1,2], Salvatierra Á[1,2], Hofland LJ[4], Luque RM[1,3], Gálvez-Moreno MA[1,2], Castaño JP[1,3]

[1] Maimonides Institute for Biomedical Research of Córdoba
[2] Reina Sofi University Hospital
[3] Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Spain
[4] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands

Lung Cancer. 2017 Jul;109:128-136. doi: 10.1016/j.lungcan.2017.05.006

ABSTRACT

Background: Lung carcinoids (LCs) are rare tumors that comprise 1-5% of lung malignancies but represent 20-30% of neuroendocrine tumors. Their incidence is progressively increasing and a better characterization of these tumors is required. Alterations in somatostatin (SST)/cortistatin (CORT) and ghrelin systems have been associated to development/progression of various endocrine-related cancers, wherein they may become useful diagnostic, prognostic and therapeutic biomarkers.

Objectives: We aimed to evaluate the expression levels of ghrelin and SST/CORT system components in LCs, as well as to explore their putative relationship with histological/clinical characteristics.

Patients and methods: An observational retrospective study was performed; 75 LC patients with clinical/histological characteristics were included. Samples from 46 patients were processed to isolate mRNA from tumoral and adjacent non-tumoral region, and the expression levels of SST/CORT and ghrelin systems components, determined by quantitative-PCR, were compared to those of 7 normal lung tissues.

Results: Patient cohort was characterized by mean age 53 ±15 years, 48% males, 34% with tobacco exposure; 71.4/28.6% typical/atypical carcinoids, 21.7% incidental tumors, 4.3% functioning tumors, 17.7% with metastasis. SST/CORT and ghrelin system components were expressed at variable levels in a high proportion of tumors, as well as in adjacent non-tumor tissues, while a lower proportion of normal lung samples also expressed these molecules. A gradation was observed from normal non-neoplastic lung tissues, non-tumoral adjacent tissue and LCs, being SST, sst4, sst5, GHS-R1a and GHS-R1b overexpressed in tumor tissue compared to normal tissue. Importantly, several SST/CORT and ghrelin system components displayed significant correlations with relevant clinical parameters, such as necrosis, peritumoral and vascular invasion, or metastasis.

Conclusion: Altogether, these data reveal a prominent, widespread expression of key SST/CORT/ghrelin system components in LCs, where they display clinical-histological correlations, which could provide novel, valuable markers for NET patient management.

Key Words: lung carcinoids; somatostatin system; ghrelin system; clinical-histological features

INTRODUCTION

Lung neuroendocrine tumors (LNETs) represent 20-30% of all neuroendocrine tumors [1-3]. The 2015 World Health Organization (WHO) classifies lung neuroendocrine tumors in low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC) and high-grade large cell neuroendocrine carcinomas (LCNEC) and small cell carcinoma (SCLC)[4]. Although these neoplasms share morphological, immunohistochemical and ultrastructural features, there are significant clinical, prognostic and therapeutic differences between subtypes. Indeed, high-grade tumors are very aggressive and display poor prognosis, while lung carcinoids (LCs, including TCs and ACs) have been less characterized, with a less standardized clinical management, apart from surgical resection or chemoradiotherapy [5, 6].

LCs display an incidence of 5-10 cases per million population/year [7, 8], and 5-15% of multiple neuroendocrine neoplasia 1 (MEN1) patients [9, 10]. Compared to other NETs, a lower proportion of LCs exhibits hormone hypersecretion, such as vasoactive intestinal peptide, adrenocorticotropic hormone (ACTH), and diuretic hormone, wherein the most common hormonal syndrome is the ectopic ACTH syndrome [11]. Paraneoplastic syndromes, including inappropriate antidiuretic hormone secretion, are even less frequent [9]. Importantly, LCs prognosis is tightly correlated to histotype, as TCs have a 5-year survival rate of 87%, presenting regional lymph node metastasis in 10-15% and distant metastases in 3-5% of cases. In contrast, ACs are more aggressive, with frequent nodal and distant metastases (20–50% respectively) and a 5-year survival of 60% [3, 12, 13]. Anyway, the only curative treatment for LCs is radical surgery [14].

The heterogeneity of these neoplasms, their different clinical behavior, and the possibility of recurrence or long-term metastasis, emphasize the importance of identifying new diagnostic and therapeutic markers, which could improve the diagnosis, prognosis and/or treatment of these patients. Accordingly, alterations in the regulatory neuroendocrine systems comprised by somatostatin (SST)/cortistatin (CORT), ghrelin and their receptors (ssts and GHSRs, respectively) have been associated to the development/ progression of various endocrine-related cancers. However, their expression has not been systematically characterized in LCs.

SST and CORT are two highly related neuropeptides that exert a plethora of physiological, often inhibitory, functions, by acting through their so-called SST receptors (sst1-5) [15-18], which regulate, among other activities, cell proliferation, differentiation, and angiogenesis [19]. They are widely distributed in normal and tumoral tissues, playing a useful role in tumor imaging (sst-scintigraphy or octreotide scan) [20]. More importantly, synthetic SST analogues (SSAs) represent a valuable therapeutic tool to treat ssts-positive tumors, to control hormone hypersecretion and tumor growth [21-24]. Additionally, ghrelin is a peptide hormone with multiple, and generally stimulatory functions, which span from hormone release to regulation of tumor cell proliferation [25, 26]. Ghrelin needs to be acylated by the enzyme ghrelin-O-acyl transferase (GOAT) [27, 28] to bind its receptor GHSR-1a [29]. The

complexity of SST/CORT and ghrelin systems has been lately expanded by the identification of additional splicing variants of ghrelin (In1-ghrelin) [30-35], ghrelin receptor (GHSR-1b) [36, 37] and sst5 (sst5TMD4 and sst5TMD5) [38-44] genes, which are overexpressed in tumoral pathologies, including gastroenteropancreatic NETs (GEP-NETs 34, 44), where they are associated with aggressive features.

The data collected in a limited number of studies on the presence and clinical implications of certain components of these systems [34, 45-54] suggest that a more detailed knowledge of the expression pattern of their components could unveil relevant implications in the diagnosis, prognosis and medical treatment of LCs. Accordingly, we implemented an exhaustive characterization of the presence/expression of the components of SST/CORT and ghrelin systems in well-characterized LCs, compared to non-tumoral adjacent tissues and normal non-neoplastic samples, and explored their putative relationship with clinical/histological characteristics.

MATERIALS AND METHODS:

Patients

The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, which was conducted in accordance with the Declaration of Helsinki and with national and international guidelines. A written informed consent was signed by every individual. Seventy-five patients with LCs who underwent surgery from 2005 to 2015 were included. Clinical records were used to collect full medical history. Endocrine-associated syndromes such as MEN or von Hippel-Lindau syndromes were excluded. LCs were evaluated and classified according to histopathology features in TC and AC. To confirm the neuroendocrine nature of all tumors, different neuroendocrine markers (including chromogranin A, synaptophysin, cytokeratin 7, cytokeratin 20, CD56, neuronal specific enolase, AE1AE3, cytokeratin, p53, glucagon, insulin, gastrin, SST, intestinal vasoactive peptide, pancreatic polypeptide and/or serotonin) were determined by immunohistochemistry following standardized protocols and evaluated by two experienced pathologists to confirm that all the included neuroendocrine tumors expressed a minimum of two different neuroendocrine markers. In addition, tissue samples were obtained from 46 of those patients and from 7 normal tissues from anonymous body organ donors. In particular, we obtained 89 formalin-fixed paraffin-embedded (FFPE) samples (46 primary tumors and 43 non-tumoral adjacent tissues) and 7 normal tissues. To ensure the appropriate identification of tumor and non-tumor adjacent areas for further RNA isolation, a comprehensive analysis of hematoxylin/eosin and immunohistochemistry sections was performed by two different experienced pathologists using conventional microscopy. Each sample was evaluated twice in order to identify, delineate and manually dissect the corresponding tissues, and when tumor and

adjacent tissue were appropriately identified, 5um slides from each paraffin-embedded tissue were cut and tumor and non-tumor adjacent regions subsequently separated for further evaluations..

RNA isolation and reverse-transcription

Total RNA from FFPE samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) according to manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [39, 55].

Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest, as previously reported. Expression levels (absolute mRNA copy number/50ng of sample) of native-ghrelin, In1-ghrelin, GOAT, GHSR-1a, GHSR-1b, SST, CORT, sst1, sst2, sst3, sst4, sst5 and sst5TMD4 were measured using previously validated primers [31, 33, 56, 57]. Samples were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as negative control. Thermal profile consisted of an initial step at 95°C for 30s, followed by 50 cycles of denaturation (95°C for 20s) and annealing/elongation (60°C for 20s), and finally, a dissociation cycle (melting curve: 55°C to 95°C, increasing 0.5°C/30s) to verify that only one product was amplified. RNA expression was adjusted by the expression of the housekeeping gen beta-actin (ACTB), whose levels were not significantly different among groups.

Immunohistochemistry (IHC) analysis

IHC analysis of GHSR-1a and sst4 was implemented in formalin-fixed, paraffin-embedded (FFPE) lung tissue samples (n=19), which included tumor and non-tumor regions from patients diagnosed with LCs, using standard procedures. The optimum antibody concentration to perform GHSR-1a and sst4 IHC analyses (1:300) using a commercially available human GHSR-1a and sst4 antibodies (Santa Cruz and AVIVA, respectively) was selected by performing a series of antibody dilution tests (1:100; 1:200; 1:300 and 1:400) in brain samples (a tissue that has been previously reported to express high levels of GHSR-1a and sst4). Two independent pathologists performed the IHC analysis of the samples following a blinded protocol. In the analysis, 1+, 2+, 3+ stand for low, moderate, and high intensities of the tumoral region staining compared to the adjacent region with non-tumor lung tissue.

Statistical analysis

Paired t-test analysis was used to compare the expression levels between LC samples and adjacent non-tumoral tissue. Non-paired t-test analysis was used to compare the expression levels between normal lung tissue and tumor or adjacent non-tumoral tissue. U-Mann Whitney tests were used to evaluate clinical-molecular relations. Chi-squared test compared categorical data. Statistical analyses were performed using SPSS statistical software v20 and GraphPad Prism v6. Data are expressed as mean±SEM. p-values<0.05 were considered statistically significant.

RESULTS

Patient population and clinical correlations

A total of 75 LCs patients were included. Demographic and clinical features are summarized in Table 1, while characteristics of the 49 tumor tissues are summarized in Table 2. Demographic and clinical characteristics were similar between TC and AC subjects. When all LC patients were considered together, age was positively correlated to second neoplasm presence

Table 1: Demographic and clinical characteristics of the patient population

General characteristic	Total n=75 (100%)	Typical n=34 (69.4%)	Atypical n=15 (30.6%)	p*
Gender				>0.05
Male	48% (36)	44.1% (15)	66.7% (10)	
Female	52% (39)	55.9% (19)	33,3% (5)	
Age	53.13±15.18 years	49.36±3.45	51.66±4.62	>0.05
Personal history of other tumors	17.4% (12)	22.6% (7)	20% (3)	>0.05
Smoke habit				>0.05
Active	34% (18)	33.3% (8)	28.6%(4)	
Ex-smoker	28.3% (15)	33.3% (8)	28.6% (4)	
No habit	37.7% (20)	33.3% (8)	28.6% (4)	
Family history of neoplasms	55.6 % (5)	66.7% (2)	33.3 % (1)	>0.05
Incidental tumor	21.7% (10)	29.4% (5)	36.4% (4)	>0.05
Pre-surgical treatment	6.1% (4)	3.2% (1)	14.3% (2)	>0.05
Clinical symptoms**				>0.05
Hemoptysis	17.9% (5)	23.5% (4)	9.1% (1)	
Cough	10.7% (3)	11.8% (2)	9.1% (1)	
Pneumonia	35.7% (10)	35.3% (6)	36.4% (4)	

**p* value refers to the comparison between typical and atypical carcinoids

** most common clinical symptoms

Table 2: Tumor sample characteristics

Tissue samples	Total (%) (46)	Typical n=22 (66.7%)	Atypical n=11 (33.3%)	p*
Primary tumor localization				
Right lung	62.2% (28)	72.7% (16)	54.5% (6)	>0.05
Left lung	37.8% (17)	27.3% (5)	45.5% (5)	>0.05
Upper lobe	25.6% (11)	18.2%(4)	36.4% (4)	>0.05
Right middle lobe	27.9% (12)	40.9% (9)	18.2% (2)	>0.05
Lower lobe	44.2% (20)	36.4% (8)	36.4% (4)	>0.05
Immunohistochemistry				
Chromogranin A	39.6% (19)	34,28% (12)	40%(6)	>0.05
Synaptophysin	31.3% (15)	28.57%(10)	28.57% (4)	>0.05
Cytokeratin 7	4.2% (2)	2.86%(1)	9.1% (1)	>0.05
Cytokeratin 20	2.1% (1)	0% (0)	7.14% (1)	>0.05
Neuronal specific enolase	22.9% (11)	17.14% (6)	21.43% (3)	>0.05
CD56	18.8% (9)	18.2% (4)	18.2% (2)	>0.05
Others**	73% (36)	62.85%(22)	71.42% (10)	>0.05
Functionality	4.3% (2)	0% (0)	8.3% (1)	>0.05
Tumor diameter (cm)	**2.72±2.05**	**2.38±0.21**	**4.81±1.36**	**0.013**
Multiple tumors	7% (5)	3% (1)	13.3% (2)	>0.05
Peri-tumoral invasion	**22.5% (9)**	**6.7% (2)**	**46.2% (6)**	**0.028**
Vascular invasion	**16.7% (4)**	**0% (0)**	**50% (4)**	**0.005**
Neural invasion	11.8% (2)	0% (0)	33.2% (2)	>0.05
Metastasis	**12.5% (5)**	**0% (0)**	**46.2% (5)**	**0,007**
Bronchial lumen localization/infiltration	80% (32)	75% (21)	66.7% (8)	>0.05
Parenchyma localization/infiltration	32.5% (13)	35.7% (10)	58.3% (7)	>0.05
Pleural localization/infiltration	7.5% (3)	10.7% (3)	0% (0)	>0.05

**p* value refers to the comparison between typical and atypical carcinoids

***Others indicate positive immunohistochemistry for (at least) one of the following neuroendocrine markers: AE1AE3, cytokeratin, p53, glucagon, insulin, gastrin, SST, intestinal vasoactive peptide, pancreatic polypeptide and/or serotonin

(p=0.006) and parenchyma localization (p=0.013), and showed a non-significant trend to correlate with vascular invasion (p=0.055). Although all tumors were positive for at least one neuroendocrine marker, these markers did not exhibit any apparent association with clinical variables. In contrast, tumor diameter was directly correlated to necrosis (p=0.016), peritumoral invasion (p=0.006) and metastasis (p=0.026). ACs exhibited significantly higher diameter (p=0.022), necrosis (p=0.013), vascular invasion (p=0.005), peritumoral tissue invasion (p=0.028), and metastasis (p<0.001) than TCs.

Histopathological characterization of LCs and non-tumoral adjacent tissue

Representative histological images of TC and ACs, adjacent non-tumor tissues and normal lungs are depicted in Figure 1. Remarkably, adjacent non-tumor tissue displayed clear signs of pathological transformation (Figure 1C) as they presented diffuse interstitial chronic inflammation characterized by lymphocytes, scattered plasma cells and occasional multinucleated giant cells, which was not observed in normal non-neoplastic lung tissues (Figure 1D).

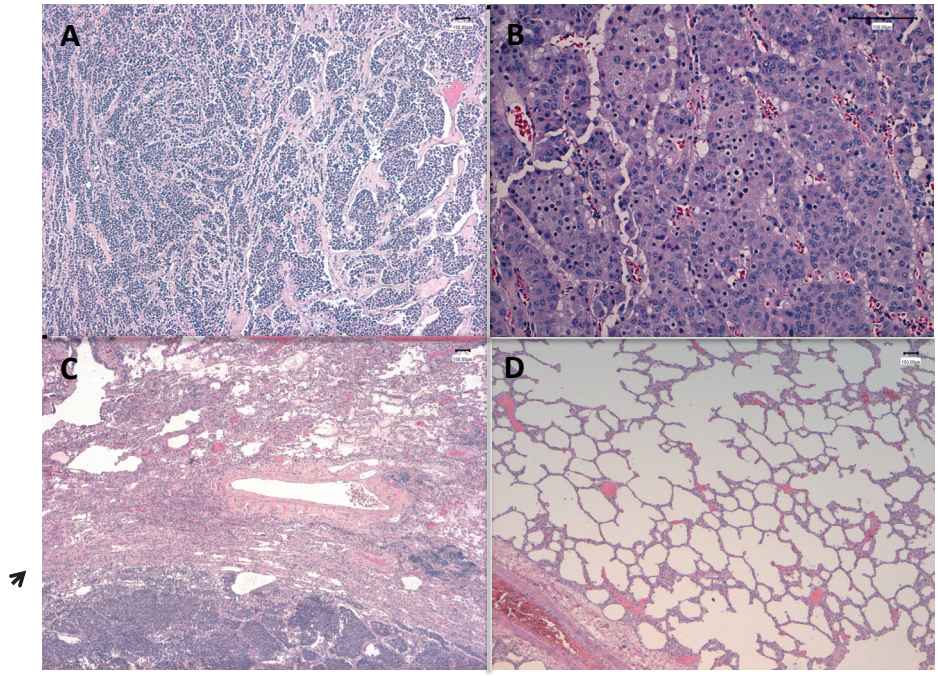


Figure 1: Histopathological evaluation of normal lung, adjacent non-tumoral tissue and LCs samples. Representative images of hematoxilin/eosin staining performed on TC (A), AC (B), adjacent non-tumoral tissue characterized by diffuse interstitial chronic inflammation with lymphocytes, scattered plasma cells and occasional multinucleated giant cells (narrows) (C) and normal lung controls (D).

Expression of SST/CORT system components in control and LC samples

SST/CORT system components were expressed in a modest proportion of normal lung samples, as determined by qPCR. Only SST and sst3 were expressed in almost 50% (3 out of 7) of normal samples, whereas other SST/CORT components were only detected in 1 or 2 samples (Figure 2A). In contrast, a high proportion of tumoral and, also, adjacent non-tumoral tissues expressed most of the SST/CORT system components (Figure 2A). Specifically, SST, sst1, sst2, sst3, sst5, and sst5TMD4 were expressed in at least 75% of adjacent non-tumoral and tumoral samples, with only sst4 being present in less than 25% of the adjacent and tumoral tissues (Figure 2A). Of note, the percentage of tumoral tissues

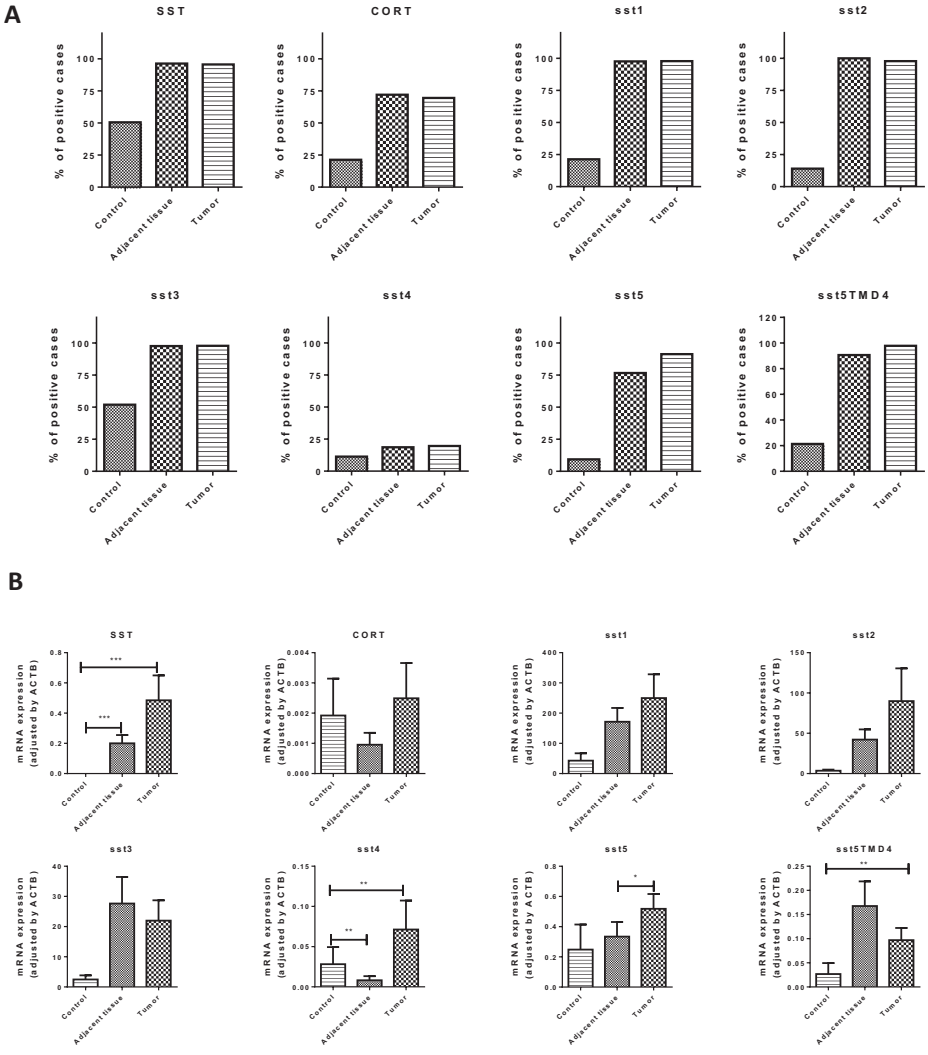


Figure 2: Presence and mRNA expression of SST/CORT system components in normal lung, adjacent non-tumoral tissue and LCs. A: The graphs indicate the percentage of samples (normal lung control, adjacent non-tumoral tissue and tumoral tissue) positive for each of the SST/CORT system components. **B:** The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in normal lung controls, adjacent non-tumoral tissue and LC samples (values are adjusted by ACTB expression). Data represent the mean±SEM. Asterisks (*, p<0.05; **, p<0.01; ***,p<0.001) indicate significant differences by paired analysis between adjacent non-tumoral and LCs and non-paired analysis between normal lung tissue and adjacent non-tumoral or tumoral tissues.

expressing the components of SST/CORT system was similar between AC and TC (p>0,05) (Suppl. Figure 1).

Expression levels of the SST/CORT system components were largely variable. In this analysis, only cases that showed detectable expression were included. SST expression levels were 100-fold higher than those of CORT, whose mRNA levels were close to the detection limits (Figure 2B). SST levels were higher in non-tumoral adjacent and tumoral tissues compared to control tissue, being this increase more pronounced in tumoral tissue (Figure 2B). In the case of the receptors, sst1 and sst2 were highly expressed, followed by sst3, while sst5, sst5TMD4 and sst4 showed lower levels. Expression of all ssts, except sst3, displayed a similar tendency, increasing progressively from control tissues to non-tumoral adjacent tissue and being apparently higher in the tumoral regions (Figure 2B). Interestingly, in tumor samples, SST expression was correlated to sst1, sst2, sst3 and sst5 expression; CORT levels were correlated to sst5 expression and, finally, sst1, sst2 and sst3 expression levels showed significant correlations (Suppl. table 1).

Expression of ghrelin system components in control and LC samples.

Ghrelin system components were also expressed in <25% of normal lung samples, as determined by qPCR (Figure 3A). In contrast, ghrelin, In1-ghrelin, GHSR-1a and GHSR-1b were expressed in at least 75% of tumoral and adjacent non-tumor tissues; while GOAT was present in less than 50% of the adjacent non-tumoral samples but in more than 75% of tumoral samples (Figure 3A). The proportion of tumoral tissues expressing ghrelin system components was not statistically significant different between AC and TC (Suppl. Figure 2). In contrast, GHSR-1a and GHSR-1b were overexpressed in tumor tissue and adjacent-non tumoral tissue compared to normal lung tissue (Figure 3B). No significant correlation was observed among the expression levels of the ghrelin system components in tumor samples (data not shown).

Immunohistochemistry (IHC) analysis

Although qPCR is a sensitive method of assaying for gene expression, we subsequently performed IHC analysis in a set of selected samples in order to validate the observed changes at the protein level, and to determine which particular cells are expressing those markers. To this end, we selected sst4 and GHSR-1a due to their clear overexpression in tumor samples. Specifically, GHSR-1a and sst4 IHC was performed on FFPE-lung carcinoids, which revealed stronger staining in tumor samples compared to non-tumor adjacent tissue (Suppl. Figure 3B, 4B). In general, IHC analysis of non-tumol adjacent tissue revealed that only few cells from pulmonary parenchyma and glandular tissue were specifically stained (Suppl. Figure 3A, 4A). However, it is worth noting that infiltrated immune cells and especially alveolar macrophages presented an intense staining in these samples. In contrast, IHC

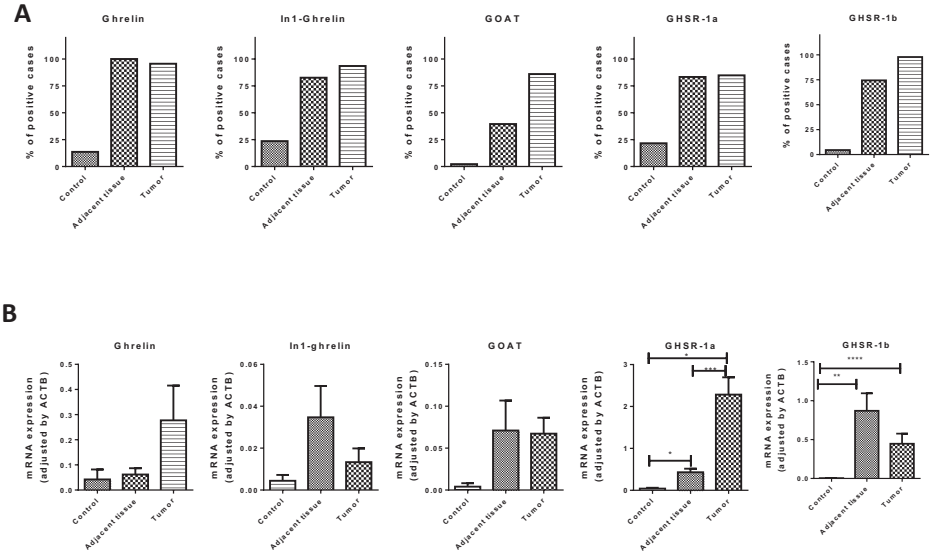


Figure 3: Presence and mRNA expression of ghrelin system components in normal lung, adjacent non-tumoral tissue and LCs. 3A: The graphs indicate the percentage of samples (normal lung control, adjacent non-tumoral tissue and tumoral tissue) positive for each of the ghrelin system components. **3B:** The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in normal lung controls, adjacent non-tumoral tissue and LC samples (values are adjusted by ACTB expression). Data represent the mean±SEM. Asterisks (*, p<0.05; **,p<0.01; ***,p<0.001) indicate significant changes by paired analysis between adjacent non-tumoral and LCs and non-paired analysis between normal lung tissue and adjacent non-tumoral or tumoral tissues.

analysis of tumor tissue revealed that GHSR-1a and sst4 were present in the vast majority of tumor cells. Interestingly, tumor samples presented variable levels of both molecules, although most samples were classified as having an intensity of 2+ or 3+ by the pathologists. Therefore, these data confirm the contention that the expression of certain components of the SST/CORT/ssts and ghrelin/GHSRs systems, and especially GHSR-1a and sst4, is clearly dysregulated in LC samples compared to non-tumor adjacent tissue.

Expression of SST/CORT and ghrelin systems components and clinical-histological characteristics in tumoral LC samples.

Correlation analyses revealed that sst3 was overexpressed in LCs of patients with tobacco smoke exposure (p<0.05), that sst5 was higher in incidental tumors, and that disease-free patients exhibited higher sst5TMD4 expression (Figure 4). Regarding the ghrelin system, ghrelin expression was correlated to vascular invasion (p=0.042) and tended to associate with bronchial localization (p=0.057) (Figure 4). Interestingly, necrotic tumors overexpressed GOAT (p<0.05) and GHSR-1a was overexpressed in tumors with parenchyma localization and in non-functional and metastatic tumors (p<0.05) (Figure 4).

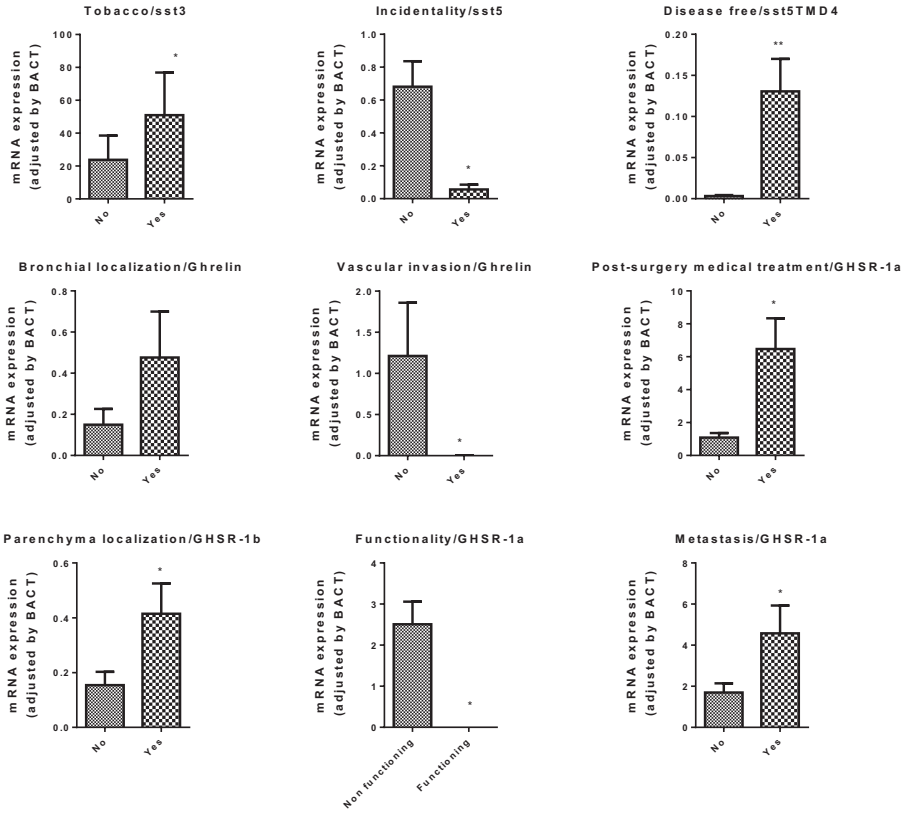


Figure 4: Correlations between epidemiological, clinical, histological and molecular parameters in LCs. The putative correlations between epidemiological, clinical, histological and molecular parameters within LC samples were assessed by U-Mann Whitney tests and asterisks (*, $p<0.05$; **, $p<0.01$) indicate significant associations.

DISCUSSION

In this study, we have comprehensively evaluated by qPCR the expression of SST/CORT and ghrelin system components in a large series of well-characterized TCs and ACs, and compared with the expression in adjacent non-tumoral and normal lung tissues. To the best of our knowledge, this study represents the first systematic characterization of the components of these regulatory systems in samples from LCs, in comparison with adjacent non-tumoral regions and normal lungs, and may therefore provide a useful overall picture of the landscape of changes associated to LCs pathology. Although several studies have explored the presence of certain SST/CORT and ghrelin systems components in LCs [45-52], their presence had never been compared to that found in adjacent non-tumoral regions or

normal lungs. In addition, specific SST/CORT and ghrelin system components displayed clinical-histological correlations in tumoral tissues, suggesting that they could provide novel, valuable markers for LC-patient management.

Consistent with previous reports showing that 75% of LCs are central (bronchial) and 25% are peripheral tumors [3], our tumor series presented a similar distribution. In addition, previous studies have reported that LCs diameter is not correlated to survival or recurrence [13]. Consistently, in our series, no direct correlation with clinical outcome was observed, although tumors >2.4cm showed higher rate of peritumoral invasion, vascular invasion and distant metastasis. In fact, no correlations between clinical, histological or immunohistochemical characteristics and survival or mortality were found herein, which is in contrast with previous reports describing some independent predictors of survival [mitotic rate, tumor size, sex [58], typical histology and lymphatic invasion [59]]. Nevertheless, our analysis revealed that age correlated directly to the presence of parenchyma localization, a second neoplasm and vascular invasion, which have not been previously reported and could suggest age as a risk factor for more aggressive tumors.

Remarkably, tumoral characteristics of TCs and ACs were markedly different. ACs exhibited significantly higher diameter, necrosis, peri-tumoral and vascular invasion and metastasis, which is consistent with higher ACs malignancy compared to TCs [60]. Similar to other reports, adjacent non-tumoral tissues exhibited signs of pathological alteration compared to normal lung samples [61-64]. Consequently, to comprehensively characterize SST/CORT and ghrelin system components expression in a large series of well-characterized TCs and ACs, and compare with their expression in adjacent non-tumoral tissues and normal lungs, we applied a qPCR-based approach as previous studies have demonstrated that mRNA levels of the components of these systems correlate well with their respective protein levels [46, 48, 49, 65, 66]. Moreover, qPCR is a more sensitive detecting method than IHC [67].

NETs are known to overexpress stss [46, 53, 54], which is important in their diagnosis and management [20, 46, 68, 69]. However, to date, only a limited number of studies have reported the expression of ssts other than sst2, with variable results, likely due to the application of different experimental approaches [19, 70, 71]. Particularly, just few studies have explored a small number of cases or only single receptor subtypes [50, 65, 67, 70, 72]. Recently, additional studies have more comprehensively characterized ssts presence on LCs [46, 48, 73]. Interestingly, the data presented in the current study reveal the prominent and widespread expression of ssts in LCs, being sst1 the most abundant, followed by sst2, sst3 and sst5, with sst4 and the truncated sst5TMD4 being the least expressed. These data agree with the majority of the previous studies [48-52, 65, 67]. Moreover, this is the first study reporting the presence of sst5TMD4 in LCs and sst5 in symptomatic patients. Although

initial studies suggested that ssts presence in LNETs could exhibit a progressive decrease from low- to high-grade forms [45]; however, our work and other studies [46] indicate that there are no major differences, suggesting that ssts could be a common hallmark of low and intermediate grade LNETs. Moreover, our results revealed, for first time, a prominent expression of SST in LCs, whereas CORT expression was comparatively negligible. Of note, expression of SST directly correlated with that of sst1, sst2, sst3 and sst5, which suggests an autocrine/paracrine SST/ssts loop capable to modulate *in situ* the progression of LCs. In addition, SST and ssts expressions displayed herein a gradation in normal lungs, non-tumoral adjacent tissue, and LCs. Of special interest is our observation that non-tumoral adjacent tissues also present a notable expression of SST and ssts, and in fact, a similar proportion of non-tumoral adjacent tissues presented ssts compared to LCs, although, in general, at lower expression levels. To further explore this notion, we selected sst4 (due to its differential distribution), to perform an IHC analysis, as it has been previously reported that specific antibodies against SST receptors allow an appropriate immunolocalization of receptor subtypes in tumor tissue with a comparable, although not quantitatively superior quality than that of qPCR (65). Results from this analysis enabled visualization of sst4 in specific cells of tumor tissue and demonstrated that non-tumor adjacent cell type (in airway epithelium and associated neuroendocrine cells, as well as in pulmonary parenchyma and associated glandular tissue) are less immunopositive that other tumor cells.

Our results also revealed a differential expression of ghrelin system components in normal lung tissues, non-tumoral adjacent tissue, and LCs. In particular, ghrelin system components were expressed at low levels in a reduced proportion of normal lungs, which is consistent with previous reports showing ghrelin expression in normal and fetal lungs [30, 47, 66, 74]; while GHSR1a was undetectable [74]. In contrast, our analysis revealed a prominent and widespread expression of ghrelin system components in LCs and adjacent non-tumoral samples. Interestingly, a higher expression levels of the canonical variants (native ghrelin and GHSR1a) is consistent with previous reports showing that ghrelin is expressed in lung tumors, regardless of their neuroendocrine phenotype, and that GHSR1a is present in well differentiated functioning and non-functioning lung NETs [29, 75]. In contrast, expression of the alternative splicing variants (In1-ghrelin/GHSR1b) is lower, and had not been reported previously. This is also the first study reporting the expression of the GOAT enzyme in a high proportion of LCs, wherein the concomitant presence of ghrelin, GOAT and GHSR1a on most LCs suggests the existence of a functional regulatory association that could be modulating the development and/or progression of this pathology. Unfortunately, no studies have yet investigated the direct effect of this in LCs, and the only report in SCLC suggests that ghrelin could inhibits cell proliferation and increases apoptosis [29], in agreement with the negative association between ghrelin and vascular invasion in our cohort. However, GOAT levels were higher in tumors with necrosis, which were the ones

with a larger size and higher capacity of peritumoral invasion and distant metastasis; this, together with the direct relationship between metastasis, requirements of post-surgical treatments, and GHSR1a expression, reinforces the idea that this system could be associated to the pathogenesis of the disease and might therefore provide novel potential diagnostic, prognostic and/or therapeutic tools in LCs.

In summary, this study provides a comprehensive primary mapping of the expression of SST/CORT and ghrelin system components (including their most relevant splicing variants), in LCs, as compared with their respective adjacent non-tumoral tissues, and with normal, non-neoplastic tissues. Our results indicate a prominent and widespread overexpression of SST/CORT and ghrelin system components in LCs and in non-tumoral adjacent tissues, wherein they could exert relevant regulatory roles, for they display changes in expression tightly linked to the degree of disease, and exhibit associations to fundamental clinical parameters. Hitherto, there has been a paucity of studies reporting clinical, biochemical, histological, immunohistochemical or molecular tumor markers that could help to accurately predict the efficacy of the medical treatment, as well as the cure or relapse rates in NETS. This goal is specially difficult and necessary in LCs, due to their rarity, high diversity and heterogeneity in terms of malignant capacity, localization, and growth pattern. In this context, our present findings may help to identify new potential diagnostic and prognostic factors, which could help to devise and implement improved therapeutic strategies, aimed at attaining a better quality of life and survival for this patients. Hence, our data provide novel information on the presence of both SST/CORT and ghrelin systems in LCs, and invite to suggest that their role in this pathology as putative molecular biomarkers and therapeutic targets for LC patients deserves further investigation.

REFERENCES

1. Gustafsson, B.I., et al., *Bronchopulmonary neuroendocrine tumors.* Cancer, 2008. **113**(1): p. 5-21.
2. Travis, W.D., *Advances in neuroendocrine lung tumors.* Ann Oncol, 2010. **21 Suppl 7**: p. vii65-71.
3. Rekhtman, N., *Neuroendocrine tumors of the lung: an update.* Arch Pathol Lab Med, 2010. **134**(11): p. 1628-38.
4. Travis, W.D., et al., *The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification.* J Thorac Oncol, 2015. **10**(9): p. 1243-60.
5. McMullan, D.M. and D.E. Wood, *Pulmonary carcinoid tumors.* Semin Thorac Cardiovasc Surg, 2003. **15**(3): p. 289-300.
6. Kosmidis, P.A., *Treatment of carcinoid of the lung.* Curr Opin Oncol, 2004. **16**(2): p. 146-9.
7. Modlin, I.M., K.D. Lye, and M. Kidd, *A 5-decade analysis of 13,715 carcinoid tumors.* Cancer, 2003. **97**(4): p. 934-59.
8. Travis, W.D., *Lung tumours with neuroendocrine differentiation.* Eur J Cancer, 2009. **45 Suppl 1**: p. 251-66.
9. Sachithanandan, N., R.A. Harle, and J.R. Burgess, *Bronchopulmonary carcinoid in multiple endocrine neoplasia type 1.* Cancer, 2005. **103**(3): p. 509-15.
10. Oberg, K., et al., *Neuroendocrine bronchial and thymic tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2012. **23 Suppl 7**: p. vii120-3.
11. Isidori, A.M., G.A. Kaltsas, and A.B. Grossman, *Ectopic ACTH syndrome.* Front Horm Res, 2006. **35**: p. 143-56.
12. Thomas, C.F., Jr., H.D. Tazelaar, and J.R. Jett, *Typical and atypical pulmonary carcinoids : outcome in patients presenting with regional lymph node involvement.* Chest, 2001. **119**(4): p. 1143-50.
13. Ferolla, P., et al., *Tumorlets, multicentric carcinoids, lymph-nodal metastases, and long-term behavior in bronchial carcinoids.* J Thorac Oncol, 2009. **4**(3): p. 383-7.
14. Swarts, D.R., F.C. Ramaekers, and E.J. Speel, *Molecular and cellular biology of neuroendocrine lung tumors: evidence for separate biological entities.* Biochim Biophys Acta, 2012. **1826**(2): p. 255-71.
15. Cordoba-Chacon, J., et al., *Truncated somatostatin receptors as new players in somatostatin-cortistatin pathophysiology.* Ann N Y Acad Sci, 2011. **1220**: p. 6-15.
16. Gahete, M.D., et al., *Somatostatin and its receptors from fish to mammals.* Ann N Y Acad Sci, 2010. **1200**: p. 43-52.
17. Gahete, M.D., et al., *Are somatostatin and cortistatin two siblings in regulating endocrine secretions? In vitro work ahead.* Mol Cell Endocrinol, 2008. **286**(1-2): p. 128-34.
18. Moller, L.N., et al., *Somatostatin receptors.* Biochim Biophys Acta, 2003. **1616**(1): p. 1-84.
19. Volante, M., F. Bozzalla-Cassione, and M. Papotti, *Somatostatin receptors and their interest in diagnostic pathology.* Endocr Pathol, 2004. **15**(4): p. 275-91.
20. Kwekkeboom, D.J., et al., *ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: somatostatin receptor imaging with (111)In-pentetreotide.* Neuroendocrinology, 2009. **90**(2): p. 184-9.
21. Kwekkeboom, D.J., et al., *Overview of results of peptide receptor radionuclide therapy with 3 radiolabeled somatostatin analogs.* J Nucl Med, 2005. **46 Suppl 1**: p. 62S-6S.

22. Theodoropoulou, M. and G.K. Stalla, *Somatostatin receptors: from signaling to clinical practice.* Front Neuroendocrinol, 2013. **34**(3): p. 228-52.
23. Ruscica, M., et al., *Somatostatin, somatostatin analogs and somatostatin receptor dynamics in the biology of cancer progression.* Curr Mol Med, 2013. **13**(4): p. 555-71.
24. Culler, M.D., et al., *Somatostatin analogs for the treatment of neuroendocrine tumors.* Cancer Metastasis Rev, 2011. **30 Suppl 1**: p. 9-17.
25. Chopin, L.K., et al., *The ghrelin axis--does it have an appetite for cancer progression?* Endocr Rev, 2012. **33**(6): p. 849-91.
26. Gahete, M.D., et al., *Ghrelin gene products, receptors, and GOAT enzyme: biological and pathophysiological insight.* J Endocrinol, 2014. **220**(1): p. R1-R24.
27. Gutierrez, J.A., et al., *Ghrelin octanoylation mediated by an orphan lipid transferase.* Proc Natl Acad Sci U S A, 2008. **105**(17): p. 6320-5.
28. Yang, J., et al., *Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone.* Cell, 2008. **132**(3): p. 387-96.
29. Cassoni, P., et al., *Ghrelin and cortistatin in lung cancer: expression of peptides and related receptors in human primary tumors and in vitro effect on the H345 small cell carcinoma cell line.* J Endocrinol Invest, 2006. **29**(9): p. 781-90.
30. Gahete, M.D., et al., *A novel human ghrelin variant (In1-ghrelin) and ghrelin-O-acyltransferase are overexpressed in breast cancer: potential pathophysiological relevance.* PLoS One, 2011. **6**(8): p. e23302.
31. Gahete, M.D., et al., *Role of ghrelin system in neuroprotection and cognitive functions: implications in Alzheimer's disease.* Peptides, 2011. **32**(11): p. 2225-8.
32. Gahete, M.D., et al., *Dietary fat alters the expression of cortistatin and ghrelin systems in the PBMCs of elderly subjects: putative implications in the postprandial inflammatory response.* Mol Nutr Food Res, 2014. **58**(9): p. 1897-906.
33. Ibanez-Costa, A., et al., *In1-ghrelin splicing variant is overexpressed in pituitary adenomas and increases their aggressive features.* Sci Rep, 2015. **5**: p. 8714.
34. Luque, R.M., et al., *In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters.* Oncotarget, 2015. **6**(23): p. 19619-33.
35. Gahete, M.D., et al., *Expression of the ghrelin and neurotensin systems is altered in the temporal lobe of Alzheimer's disease patients.* J Alzheimers Dis, 2010. **22**(3): p. 819-28.
36. Korbonits, M., et al., *The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors.* J Clin Endocrinol Metab, 2001. **86**(2): p. 881-7.
37. Jeffery, P.L., A.C. Herington, and L.K. Chopin, *Expression and action of the growth hormone releasing peptide ghrelin and its receptor in prostate cancer cell lines.* J Endocrinol, 2002. **172**(3): p. R7-11.
38. Duran-Prado, M., et al., *The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells.* Oncogene, 2012. **31**(16): p. 2049-61.
39. Duran-Prado, M., et al., *Identification and characterization of two novel truncated but functional isoforms of the somatostatin receptor subtype 5 differentially present in pituitary tumors.* J Clin Endocrinol Metab, 2009. **94**(7): p. 2634-43.

40. Durán-Prado, M., et al., *A potential inhibitory role for the new truncated variant of somatostatin receptor 5 (sst5TMD4) in pituitary adenomas poorly responsive to somatostatin analogs.* J Clin Endocrinol Metab, 2010. **95**(5): p. 2497-502.
41. Luque, R.M., et al., *Truncated somatostatin receptor variant sst5TMD4 confers aggressive features (proliferation, invasion and reduced octreotide response) to somatotropinomas.* Cancer Lett, 2015. **359**(2): p. 299-306.
42. Mole, D., et al., *The expression of the truncated isoform of somatostatin receptor subtype 5 associates with aggressiveness in medullary thyroid carcinoma cells.* Endocrine, 2015. **50**(2): p. 442-52.
43. Puig-Domingo, M., et al., *The Truncated Isoform of Somatostatin Receptor5 (sst5TMD4) Is Associated with Poorly Differentiated Thyroid Cancer.* PLoS One, 2014. **9**(1): p. e85527.
44. Sampedro-Nunez, M., et al., *Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors.* Oncotarget, 2015. **7**(6): p. 6593-608.
45. Righi, L., et al., *Somatostatin receptor tissue distribution in lung neuroendocrine tumours: a clinicopathologic and immunohistochemical study of 218 'clinically aggressive' cases.* Ann Oncol, 2010. **21**(3): p. 548-55.
46. Kanakis, G., et al., *Expression of Somatostatin Receptors 1-5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role.* Neuroendocrinology, 2015. **101**(3): p. 211-22.
47. Volante, M., et al., *Ghrelin expression in fetal, infant, and adult human lung.* J Histochem Cytochem, 2002. **50**(8): p. 1013-21.
48. Tsuta, K., Wistuba, II, and C.A. Moran, *Differential expression of somatostatin receptors 1-5 in neuroendocrine carcinoma of the lung.* Pathol Res Pract, 2012. **208**(8): p. 470-4.
49. Kaemmerer, D., et al., *Somatostatin receptors in bronchopulmonary neuroendocrine neoplasms: new diagnostic, prognostic, and therapeutic markers.* J Clin Endocrinol Metab, 2015. **100**(3): p. 831-40.
50. Lupp, A., et al., *Reassessment of sst(5) somatostatin receptor expression in normal and neoplastic human tissues using the novel rabbit monoclonal antibody UMB-4.* Neuroendocrinology, 2011. **94**(3): p. 255-64.
51. Lupp, A., et al., *Reassessment of sst3 somatostatin receptor expression in human normal and neoplastic tissues using the novel rabbit monoclonal antibody UMB-5.* Neuroendocrinology, 2012. **96**(4): p. 301-10.
52. Lupp, A., F. Nagel, and S. Schulz, *Reevaluation of sst(1) somatostatin receptor expression in human normal and neoplastic tissues using the novel rabbit monoclonal antibody UMB-7.* Regul Pept, 2013. **183**: p. 1-6.
53. Castano, J.P., et al., *Gastrointestinal neuroendocrine tumors (NETs): new diagnostic and therapeutic challenges.* Cancer Metastasis Rev, 2014.
54. Oberg, K., et al., *Molecular pathogenesis of neuroendocrine tumors: implications for current and future therapeutic approaches.* Clin Cancer Res, 2013. **19**(11): p. 2842-9.
55. Martinez-Fuentes, A.J., et al., *Ghrelin is produced by and directly activates corticotrope cells from adrenocorticotropin-secreting adenomas.* J Clin Endocrinol Metab, 2006. **91**(6): p. 2225-31.
56. Gahete, M.D., et al., *Expression of Somatostatin, cortistatin, and their receptors, as well as dopamine receptors, but not of neprilysin, are reduced in the temporal lobe of Alzheimer's disease patients.* J Alzheimers Dis, 2010. **20**(2): p. 465-75.
57. Taboada, G.F., et al., *Quantitative analysis of somatostatin receptor subtype (SSTR1-5) gene expression levels in somatotropinomas and non-functioning pituitary adenomas.* Eur J Endocrinol, 2007. **156**(1): p. 65-74.

58. Beasley, M.B., et al., *Pulmonary atypical carcinoid: predictors of survival in 106 cases.* Hum Pathol, 2000. **31**(10): p. 1255-65.
59. Rea, F., et al., *Outcome and surgical strategy in bronchial carcinoid tumors: single institution experience with 252 patients.* Eur J Cardiothorac Surg, 2007. **31**(2): p. 186-91.
60. Fisseler-Eckhoff, A. and M. Demes, *Neuroendocrine tumors of the lung.* Cancers (Basel), 2012. **4**(3): p. 777-98.
61. Korner, M.U., et al., *Wild-type and splice-variant secretin receptors in lung cancer: overexpression in carcinoid tumors and peritumoral lung tissue.* Mod Pathol, 2008. **21**(4): p. 387-95.
62. Koomagi, R., et al., *Expression of resistance-related proteins in tumoral and peritumoral tissues of patients with lung cancer.* Cancer Lett, 1996. **110**(1-2): p. 129-36.
63. Li, S.X., et al., *Expression of survivin and caspase 3 in oral squamous cell carcinoma and peritumoral tissue.* Asian Pac J Cancer Prev, 2012. **13**(10): p. 5027-31.
64. Haybaeck, J., et al., *STAT-1 expression in human glioblastoma and peritumoral tissue.* Anticancer Res, 2007. **27**(6B): p. 3829-35.
65. Mizutani, G., et al., *Expression of Somatostatin Receptor (SSTR) Subtypes (SSTR-1, 2A, 3, 4 and 5) in Neuroendocrine Tumors Using Real-time RT-PCR Method and Immunohistochemistry.* Acta Histochem Cytochem, 2012. **45**(3): p. 167-76.
66. Ghelardoni, S., et al., *Ghrelin tissue distribution: comparison between gene and protein expression.* J Endocrinol Invest, 2006. **29**(2): p. 115-21.
67. Papotti, M., et al., *Expression of somatostatin receptor types 2, 3 and 5 in biopsies and surgical specimens of human lung tumours. Correlation with preoperative octreotide scintigraphy.* Virchows Arch, 2001. **439**(6): p. 787-97.
68. Lamberts, S.W., J.C. Reubi, and E.P. Krenning, *Somatostatin receptor imaging in the diagnosis and treatment of neuroendocrine tumors.* J Steroid Biochem Mol Biol, 1992. **43**(1-3): p. 185-8.
69. Eriksson, B., et al., *The use of new somatostatin analogues, lanreotide and octastatin, in neuroendocrine gastro-intestinal tumours.* Digestion, 1996. **57 Suppl 1**: p. 77-80.
70. Kimura, N., et al., *Immunohistochemical expression of somatostatin type 2A receptor in neuroendocrine tumors.* Clin Cancer Res, 1999. **5**(11): p. 3483-7.
71. Janson, E.T., et al., *Somatostatin receptor scintigraphy during treatment with lanreotide in patients with neuroendocrine tumors.* Nucl Med Biol, 1999. **26**(8): p. 877-82.
72. Reubi, J.C., et al., *Subcellular distribution of somatostatin sst2A receptors in human tumors of the nervous and neuroendocrine systems: membranous versus intracellular location.* J Clin Endocrinol Metab, 2000. **85**(10): p. 3882-91.
73. Kaemmerer, D., et al., *Correlation of monoclonal and polyclonal somatostatin receptor 5 antibodies in pancreatic neuroendocrine tumors.* Int J Clin Exp Pathol, 2013. **6**(1): p. 49-54.
74. Gnanapavan, S., et al., *The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans.* J Clin Endocrinol Metab, 2002. **87**(6): p. 2988.
75. Arnaldi, G., et al., *Cyclical Cushing's syndrome in a patient with a bronchial neuroendocrine tumor (typical carcinoid) expressing ghrelin and growth hormone secretagogue receptors.* J Clin Endocrinol Metab, 2003. **88**(12): p. 5834-40.

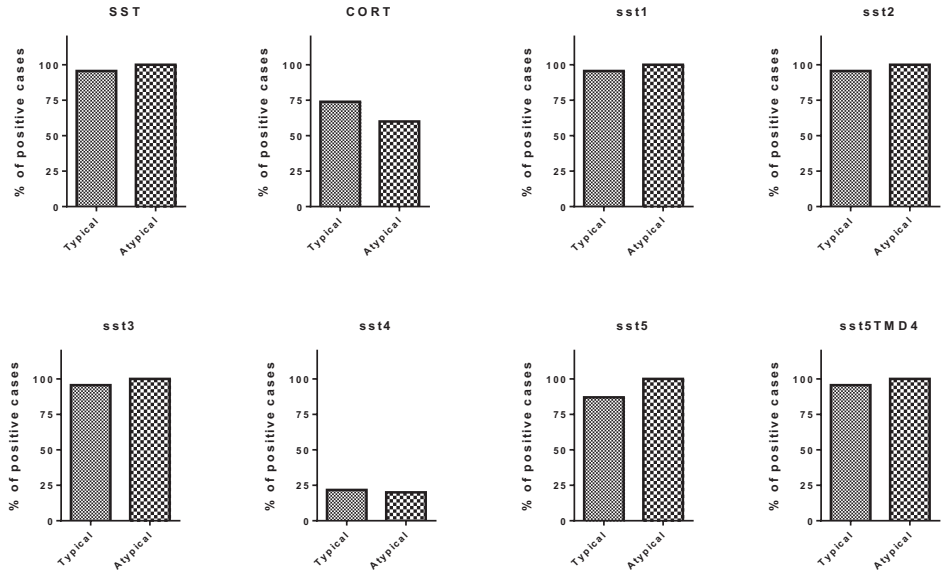
SUPPLEMENTAL DATA

Supplemental Table 1: SST/CORT system components correlations in tumor tissue

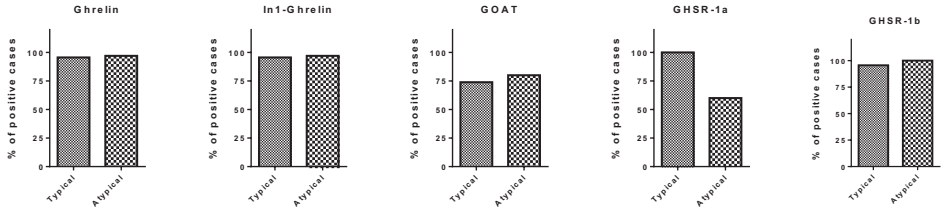
	sst1	sst2	sst3	sst4	sst5	sst5TMD4
SST	0.522	0.613	0.364		0.392	
CORT					0.430	
sst1		0.838	0.784			
sst2			0.701			

p<0.05 p<0.001 p<0.0001

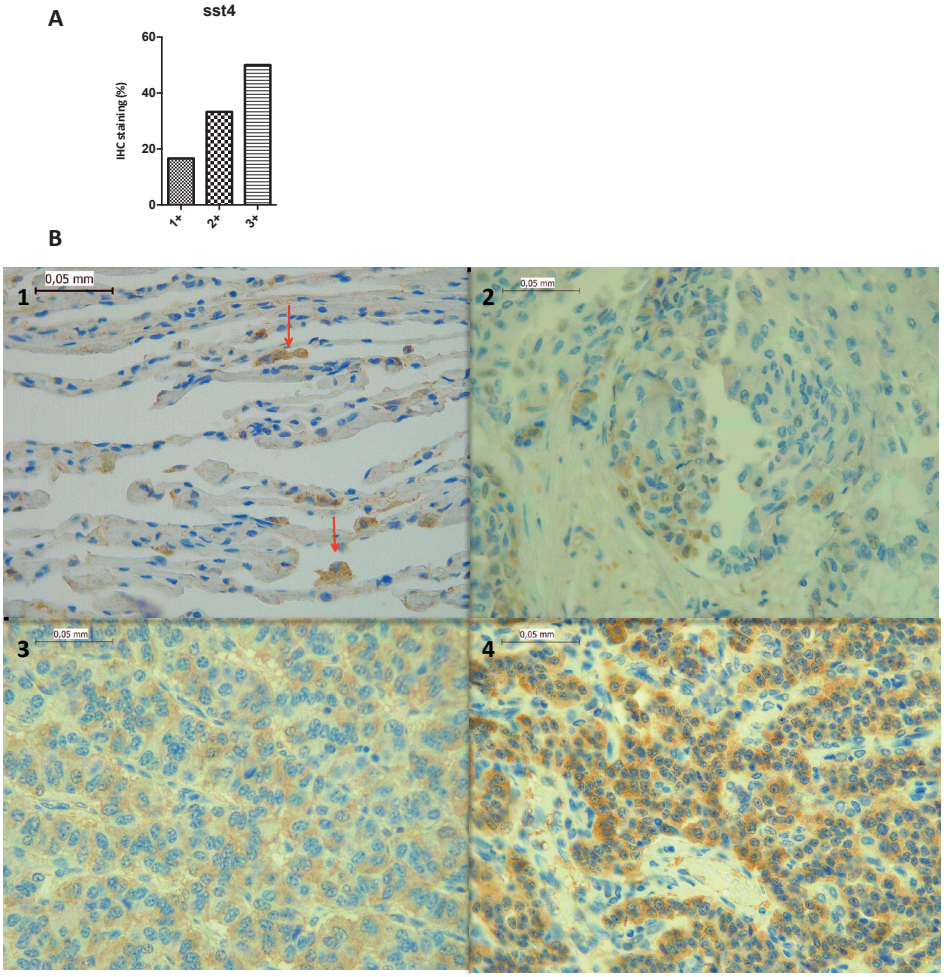
p value refers to the comparison between SST system components



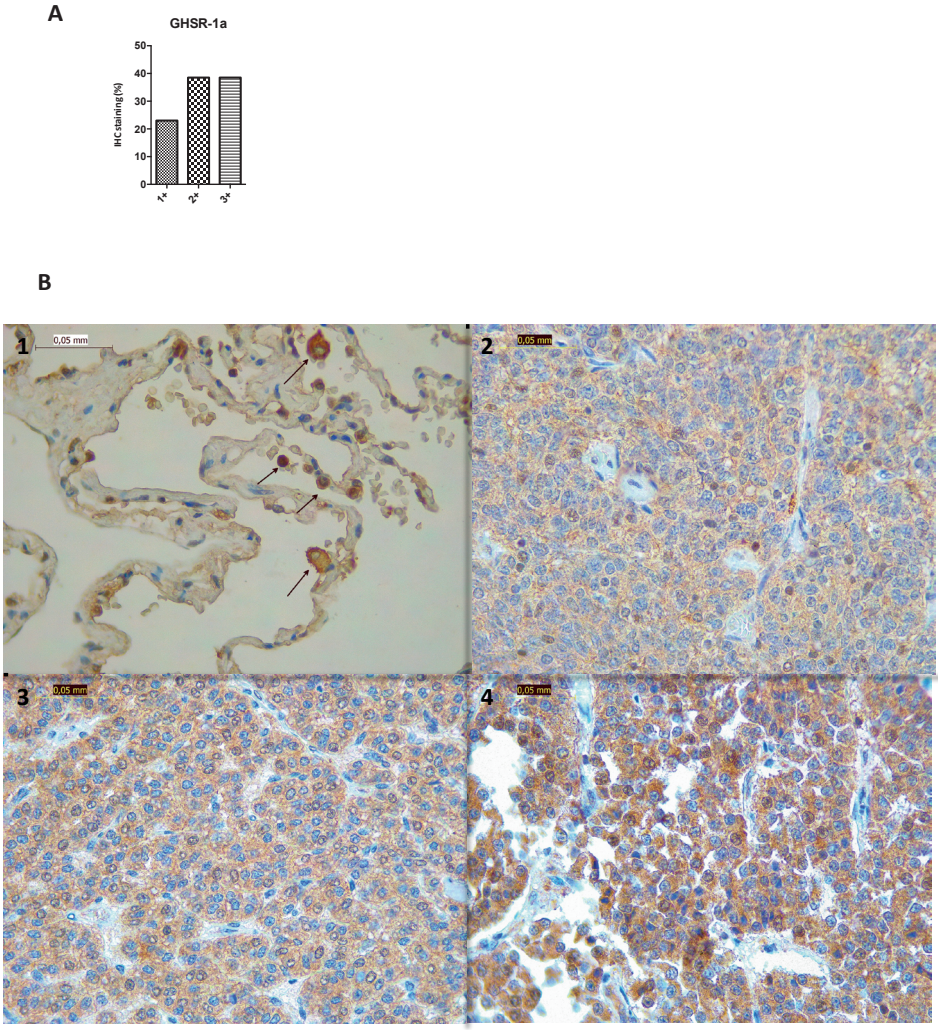
Supplemental Figure 1: Presence of SST/CORT system components in TC and AC. The graphs indicate the percentage of positive samples for each of the SST/CORT system components.



Supplemental Figure 2: Presence of ghrelin system components in TC and AC. The graphs indicate the percentage of positive samples for each of the ghrelin system components.



Supplemental Figure 3: Presence of sst4 by IHC in TC and AC. The expression of sst4 by immnuno-histochemistry was determined in adjacent non-tumor tissue and LCs samples. **A:** Percentage of cases with 1+, 2+, 3+ staining, which represent low, moderate, and high intensities of the tumor region compared to the adjacent non-tumor tissue. **B**: Representative images of sst4 staining performed on non-tumor adjacent tissue (1), 1+ (2), 2+ (3) and 3+ (4) tumor LCs samples. The arrows represent isolated alveolar macrophages with positive IHC staining.



Supplemental Figure 4: Presence of GHSR1a by IHC in TC and AC. The expression of sst4 by immnunohistochemistry was determined in adjacent non-tumor tissue and LCs samples. **A:** Percentage of cases with 1+, 2+, 3+ staining, which represent low, moderate, and high intensities of the tumor region compared to the adjacent non-tumor tissue. **B**: Representative images of sst4 staining performed on non-tumor adjacent tissue (1), 1+ (2), 2+ (3) and 3+ (4) tumor LCs samples. The arrows represent isolated pneumocytes and alveolar macrophages with positive IHC staining.

Chapter 3

Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors

Herrera-Martínez AD[1,2], Gahete MD[1,3]Pedraza-Arevalo S[1,3] Sánchez-Sánchez R[1,2], Ortega-Salas R[1,2], Serrano-Blanch R[1,2], Luque RM[1,3], Gálvez-Moreno MA[1,2], Castaño JP[1,3]

[1] Maimonides Institute for Biomedical Research of Córdoba
[2] Reina Sofi University Hospital
[3] Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Spain

Endocrine. 2018 Feb;59(2):426-437. doi: 10.1007/s12020-017-1482-3.

ABSTRACT

Purpose: Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) comprise a heterogeneous group of malignancies often presenting with metastasis at diagnosis and whose clinical outcome is difficult to predict. Somatostatin (SST) analogs (SSAs) provide a valuable pharmacological tool to palliate hormonal symptoms, and control progression in some NETs. However, many patients do not respond to SSAs or develop resistance, and there are many uncertainties regarding pathophysiology of SST and its receptors (sst1-sst5) in GEP-NETs.

Methods: The expression of SST system components in GEP-NETs was determined, compared with that of non-tumor adjacent and normal tissues and correlated with clinical and histological characteristics. Specifically, 58 patients with GEP-NETs and 14 normal samples were included. Cell viability in NET cell lines was determined in response to specific SSAs.

Results: Normal samples and non-tumor adjacent tissues presented a similar expression profile, with appreciable expression of sst2 and sst3, and a lower expression of the other receptors. In contrast, cortistatin, sst1, sst4 and sst5 were overexpressed in tumors, while sst3 and sst4 seemed overexpressed in less differentiated tumors. Some SST system components were related to vascular/nerve invasion and metastasis. *In vitro*, sst1 and sst3 agonists reduced viability in BON-1 cells, while they, similar to octreotide and pasireotide, increased viability in QGP-1 cells.

Conclusions: These results provide novel information on SST system pathophysiology in GEP-NETs, including relevant associations with clinical-histological parameters, which might help to better understand the intrinsic heterogeneity of NETs and to identify novel biomarkers and/or targets with potential prognostic and/or therapeutic value for GEP-NETs patients.

Key words: Carcinoids, somatostatin analogs (SSAs), sst1, sst3, prognosis, invasion, metastasis.

INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogeneous group of malignancies arising from neuroendocrine cells of the diffuse endocrine system, which present a unique rising incidence in the last decades: from 1.09 (in 1973) to 6.98 (in 2017) new cases per 100,000 inhabitants annually [1]. Indeed, the Surveillance, Epidemiology and End Results (SEER) further support this contention by showing an increase in the incidence of GEP-NETs of all origins except to appendix NETs [2]. Gastroenteropancreatic NETs (GEP-NETs) originate from the pancreas or the intestinal tract and represent approximately 65% of all NETs and 2% of all gastroenteropancreatic malignancies [3, 4]. These neoplasms include functioning and non-functioning tumors, depending on their capacity to secrete peptide hormones, and can occur sporadically or as a result of hereditary predisposition syndromes such as multiple endocrine neoplasia type 1 (MEN-1) or Von Hippel–Lindau's disease (VHL), with clinical onset occurring earlier in patients with genetic predisposition [5]. Although some prognostic factors include primary tumor localization, histological differentiation, Ki67 index, platelets, and lactate dehydrogenase (LDH) levels [6], their clinical outcome is mostly unpredictable, in that even well-differentiated low-grade tumors can display an aggressive behavior [3]. Furthermore, in about 30% of NETs the primary tumor cannot be identified [6], and metastatic disease is frequently found at diagnosis, especially in non-functioning tumors, ranging from 27 to 73% depending on the series, with an overall survival rates varying from 30 to 90% at 5 years [5, 7, 8]. As a result, most tumors are diagnosed at an advanced stage of disease, wherein the only curative therapeutic option is surgery. For this reason, identification of novel, early diagnostic biomarkers, and development of new targeted medical treatments has gained scientific and clinical interest over the past recent years [9].

Somatostatin (SST), cortistatin (CORT), and their 5 receptors ss1-sst5 (i.e. the SST system) are widely expressed in multiple tissues, including the gastrointestinal tract (GIT) [10, 11]. In fact, based on their capacity to inhibit endocrine secretions from the GIT, synthetic SST analogs (SSAs) have long been used to palliate hormonal symptoms in NETs. Moreover, SSAs can also control disease progression in well-differentiated, metastatic midgut NETs and in moderately differentiated, metastatic nonfunctioning GEP-NETs [12]. Thus, the PROMID study showed that SSAs can delay tumor progression in both functionally active and inactive NETs [13], while the CLARINET study reported an increase in median progression-free survival in SSA-treated patients [14]. Nevertheless, many tumors, despite bearing sst, do not respond or eventually develop resistance to SSA treatments.

The antisecretory and antitumor actions of SSAs are mediated through the SST receptors (sst1-5), a family of five G-protein-coupled receptors that are widely distributed throughout the body and are present in most GEP-NETs [15-17]. Some studies suggest that the

antiproliferative effects of SSAs are associated to their affinity for sst2 [18]. SSA treatment commonly exhibit a good safety profile [3] but after long-term tachyphylaxis may occur, which has been associated to a possible loss of sst2 availability following receptor internalization and degradation [19]. In addition, presence of sst5TMD4, a truncated splice variant of the sst5 subtype that interacts with sst2 and disrupts its signaling, has been associated with increased aggressiveness in pancreatic NETs and other hormone-dependent tumors [20, 21]. However, the exact mechanisms by which SST and SSAs exert their effects on NET cells are complex and not fully understood [3]. In fact, although various studies have examined the presence of SST system components in NETs, the clinical implications of all their members are not completely elucidated [22-24].

The intrinsic heterogeneity of NETs coupled to the their unpredictable behavior and prognosis complicates their clinical management, particularly owing to the lack of sufficient and reliable biomarkers to predict medical treatment response and patient prognosis. Accordingly, in this study we aimed to systematically and comprehensively determine the precise expression of SST system components in GEP-NETs, compare to non-tumor adjacent tissue and normal control tissue, and correlate them with clinical, functional and histological characteristics.

MATERIALS AND METHODS

Patients and samples

The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, which was conducted in accordance with the Declaration of Helsinki and according to national and international guidelines. Every individual or family member signed a written informed consent before inclusion into the study. Data from 58 patients with GEP-NETs who underwent surgery at the Hospital from 2005 to 2015 were collected (demographic and clinical characteristics of the included patients are summarized in Table 1). As well, 14 normal control tissues were also included (four whole pancreas, three stomach, four midgut and three foregut samples). Patients with neurofibromatosis, multiple endocrine neoplasia type 1, Von Hippel-Lindau or any endocrine syndrome were excluded from the present study. Clinical records were used to collect full medical history of all patients. GEP-NETs were classified according to histopathological features as: well-differentiated NETs (G1), well-differentiated neuroendocrine carcinomas (G2), and poorly differentiated neuroendocrine carcinomas (G3). Cell survival was determined by counting Ki67 positive cells by experienced pathologists [25]. After surgery, if residual disease or relapse was observed, adjuvant treatment with SSAs or chemotherapy was prescribed; however, only two cases received pre-surgery treatment with SSAs. From all these subjects, we obtained 130 formalin-fixed

Table 1: General characteristics of GEP-NETs patients

General characteristic		% (n)
Gender		
	Male	48,3% (28)
	Female	51,7% (30)
Age		56,41±15,6 years
Personal history of other tumors		11,7%
Smoke habit		
	Active	57,7% (15)
	Ex-smoker	15,4% (4)
	No habit	26,9% (7)
Family history of neoplasms		45,5% (10)
Incidental tumor		35% (14)
Functionality		43,9% (18)
Pre-surgical treatment		14% (7)
SSA pre-surgical treatment		4% (2)

paraffin-embedded samples (58 tumor samples, 58 non-tumor adjacent tissue samples and 14 normal control tissues). In order to ensure the appropriate identification of relevant areas of tumor and non-tumor tissues to carry out the RNA isolation, a comprehensive analysis of hematoxylin and eosin (H&E) sections was performed by experienced pathologists.

RNA isolation and reverse-transcription

Total RNA from formalin fixed paraffin-embedded (FFPE) samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [26, 27].

Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Specifically, expression levels (absolute mRNA copy number/50 ng of sample) of SST, cortistatin (CORT), sst1, sst2, sst3, sst4, sst5 and sst5TMD4 were measured using previously validated primers [28, 29]. Briefly, samples derived from human GEP-NET tissues were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as a negative control. Thermal profile consisted of an initial step at 95°C for 30 seconds, followed by 50 cycles of denaturation (95°C for 20s) and annealing/

elongation (60°C for 20s), and finally, a dissociation cycle (melting curve; 55°C to 95°C, increasing 0, 5°C/30 s) to verify that only one product was amplified. RNA expression was adjusted by the expression of 18S.

Immunohistochemistry (IHC) analysis

IHC analysis of sst1, sst2 and sst5 was implemented in formalin-fixed, paraffin-embedded (FFPE) tissue samples, which included tumor and non-tumor regions from patients diagnosed with GEP-NETs, using standard procedures. The optimum antibody concentrations to perform the IHC analyses (1:300) using commercially available antibodies against human sst1, sst2 and sst5 (respectively, UMB7, UMB1 and UMB4 from Abcam, Cambridge, UK) were selected by performing a series of antibody dilution tests (1:100; 1:200; 1:300 and 1:400) in brain samples (a tissue that has been previously reported to express high levels of somatostatin receptors [30, 31]). Independent pathologists performed the IHC analysis of the samples following a blinded protocol. In the analysis, negative, 1+, 2+, 3+ stand for absent, low, moderate, and high intensities of the tumor region staining compared to the adjacent region with non-tumor lung tissue.

Cell cultures

The human NET model cell lines BON-1 and QGP-1 were used in this study [20, 32]. Particularly, the human pancreatic neuroendocrine tumor BON-1 cell line was established from a lymph node metastasis of a human functional pancreatic NET BON-1 (The University of Texas Medical Branch, Galveston, Tex., USA), while the cell line QGP-1 was initially obtained from primary pancreas NET tissue obtained from a 61-year-old male [33]. Both cell lines were checked for mycoplasma contamination by PCR as previously reported [34]. BON-1 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-F12; Life Technologies, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Madrid, Spain), 1% glutamine (Sigma-Aldrich) and 0.2% antibiotic (Gentamicin/Amphotericin B; Life Technologies). QGP-1 cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland), supplemented with 10% FBS, 1% glutamine and 0.2% antibiotic. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium; cell viability always exceeded 85%. Both cell lines were cultured at 37°C in a 5% CO_2 incubator. All cell line experiments were performed at least four times.

Drugs and reagents

The sst1 agonist (IPSEN Bioscience) was diluted in medium 5% FBS prior to use until obtaining final concentration of 10^{-6} M; sst3 agonist (IPSEN Bioscience), octreotide (Novartis Pharmaceuticals Corporation), and pasireotide (Novartis Pharmaceuticals Corporation), were diluted in medium 5% FBS prior to use until obtaining final concentration of 10^{-7} M. Paclitaxel 10^{-7} M (Sigma-Aldrich) was used as positive control. The tested concentrations

were chosen based on the reported dose-dependent effects and sst1-5 subtype-binding affinities of somatostatin analogs, according to previously published data [35-41].

Cell viability assay

Cells were plated in 96-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment (5.000 cells/well). Twenty-four h later, serum-free medium was added during 24 h. Basal and 24h cell viability was determined using Alamar-Blue reagent. Specifically, the day of measurement, cells were incubated for 3 h in 10% Alamar-Blue/serum-free medium and then, Alamar-Blue reduction was measured in a FlexStation3 system (Molecular Devices) plate reader, exciting at 560 nm and reading at 590 nm. After this, the appropriate treatments (sst1 and sst3 agonists, octreotide and pasireotide) were added to wells in 5% FBS medium. In all instances, cells were seeded per quadruplicate and all assays were repeated a minimum of four times.

Statistical analysis

Paired t-test analysis was used to compare the expression levels between GEP-NETs samples and adjacent non-tumor tissue. Non-paired t-test analysis was used to compare the expression levels between normal lung tissue and GEP-NETs samples or adjacent non-tumor tissue. Mann-Whitney U test was used to evaluate clinical-molecular relations within GEP-NETs samples. Chi-squared test was used to compare categorical data. In functional experiments, results are expressed as percentage of control (non-treated cells). Cell survival rate compared to control was assessed by multiple comparison tests (One-way ANOVA followed by Dunnet post-hoc test). All statistical analyses were performed using SPSS statistical software version 20 and Graph Pad Prism version 6. Data are expressed as mean ± SEM. P-values < 0.05 were considered statistically significant.

RESULTS

A total of 58 patients with GEP-NETs and 14 normal control samples were included in the study. Clinical features of patients are summarized in Table 1, and tumor characteristics in Table 2. Twenty-eight patients (48.3%) had pancreatic NETs and 51.7% had gastrointestinal NETs; 43.9% were functioning tumors. Quantification of Ki-67 and mitotic index was available at diagnosis in only 75% of samples, 71% of samples were low or intermediate grade tumors. Interestingly, 51.7% of patients had metastasis at diagnosis, relapsed disease was observed in 41.2% of patients, and the mortality rate reached 24.5%.

Table 2: Tumor sample characteristics

Characteristic		n (%)
Tissue samples		
	Primary tumor	58
	Non-tumor adjacent tissue	58
	Normal tissue	14
Primary tumor localization		
	Pancreas	48,3% (28)
	Stomach	5,2% (3)
	Small bowel	22,4% (13)
	Colon and rectum	24,1% (14)
Maximal tumor diameter		3,16±2,09 cm
Necrosis	<10%	14,3% (1)
	10-20%	28,6% (2)
	21-30%	42,9% (3)
	>30%	14,3% (1)
Depth of infiltration (gastrointestinal NETs)		
	Submucosa	4% (1)
	Mucosa	4% (1)
	Muscular	44%(11)
	Serosa	48%(12)
Multiple tumors		7,1% (2)
Peri-tumor tissue invasion		66,7% (36)
Vascular invasion		36% (18)
Neural invasion		32,7% (16)
Metastasis		51,7% (30)
Metastasis localization		
	Liver	10% (3)
	Spleen	3,3% (1)
	Lymphatic nodules	27,6% (16)
	Multiple	29,9% (10)
Grading (WHO 2010 criteria)		
	Low	32,8% (19)
	Intermediate	37,9% (22)
	High	5,2% (3)
	Unknown	24,1% (14)
Post-surgical treatment		40,4% (19)
Relapsed disease		41,2% (21)
Disease free		61,4% (27)
New surgery requirements		23,1% (9)

Histopathological characterization of GEP-NETs and non-tumor adjacent tissue

Primary tumor samples were delimited from the non-tumor adjacent tissues after the evaluation of two experienced pathologists using histology and immunohistochemistry (Figure 1).

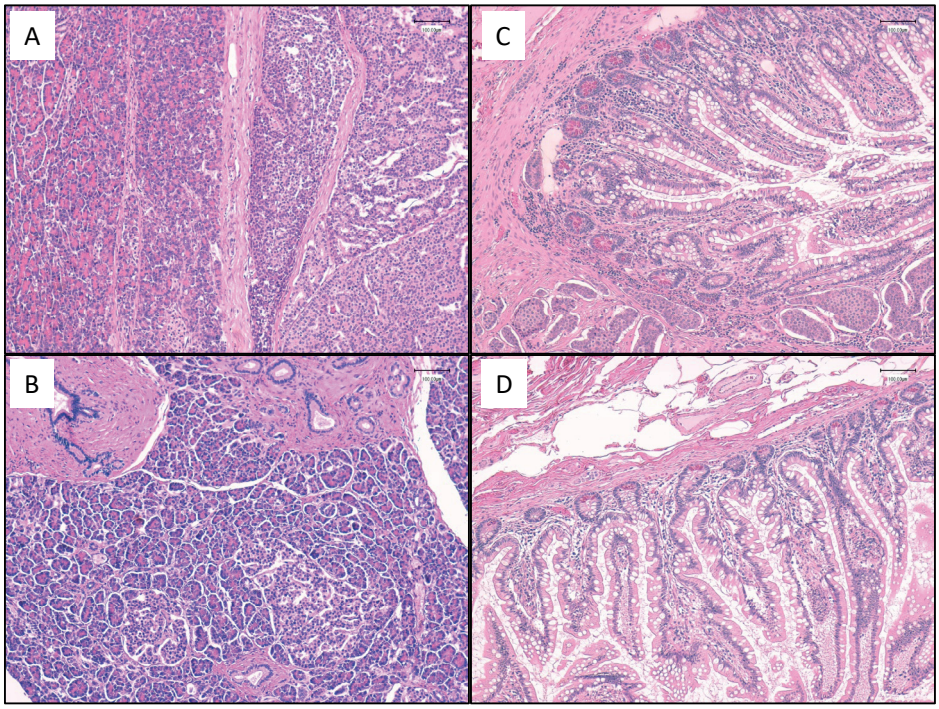


Figure 1: Representative images of pancreatic and intestinal NETs and control tissues. All images were taken from hematoxylin-eosin stained specimens at 100X. A) Pancreatic NET and adjacent tissue. B) Normal pancreas. C) Small intestine NET and adjacent tissue. D) Normal intestine. Scale bar is included in each image.

Expression of SST system components in control and GEP-NETs samples

Using a yes/no scale, SST system components were expressed in a high percentage of normal (control) GEP samples as determined by qPCR, especially SST, sst2, sst3 and sst5TMD4, which were expressed in more than 80% of the samples; while CORT was expressed in less than 30% of samples (Supplemental Figure 1). Consistently, all system components, except sst4, were expressed in at least 60% of adjacent non-tumor and tumor tissues. Instead CORT, sst1, sst4 and sst5 were present in a higher proportion of GEP-NETs samples compared to adjacent tissues, although the differences were not statistically significant (Supplemental Figure 1).

Normal tissue (control) samples and non-tumor adjacent tissues presented a similar profile of mRNA expression levels, with a considerable expression of sst2 and sst3 and a lower

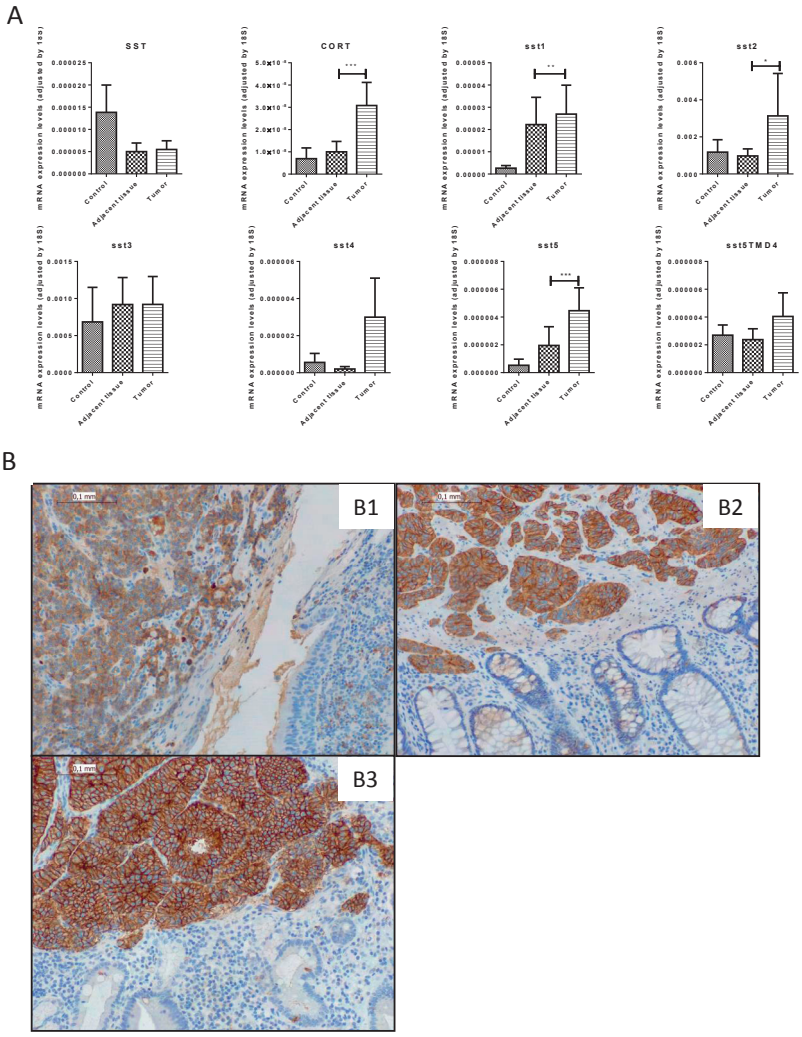


Figure 2: Presence and expression of SST/CORT system components in normal samples, adjacent non-tumor tissue and GEP-NETs. A) The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in normal control samples, adjacent non-tumor tissue and GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean±SEM. Asterisks (*, p<0.05; **, p<0.01; ***, p<0.001) indicate significant changes by paired analysis between adjacent non-tumor and GEP-NETs and non-paired analysis between normal tissue and adjacent non-tumor or tumor tissues. B) **Immunohistochemical analysis of somatostatin** receptors sst1, sst2 and sst5 **in adjacent non-tumor tissue and GEP-NETs.** The presence of sst1, sst2 and sst5 by immunohistochemistry using specific antibodies was determined in a subset of samples, which included tumor and non-tumor regions from patients diagnosed with GEP-NETs. Representative images from pieces stained with sst1, sst2 and sst5 are depicted in B1, B2 and B3, respectively. This analysis revealed that somatostatin receptors were present in the vast majority of tumor cells compared with non-tumor adjacent tissue, with different grades of staining.

expression of the rest of receptors (sst1, sst4 and sst5) and the ligands (SST and CORT). Interestingly, CORT and the receptors sst1, sst2, and sst5 were clearly overexpressed in tumor tissues compared with adjacent non-tumor tissue samples (Figure 2). Expression of sst3 and sst4 tended to increase in GEP-NETs according to their grade, while the opposite trend (decreasing expression levels with increasing tumor grade) was observed for SST, sst1, sst2, sst5, and sst5TMD4 (Supplemental Figure 2). When tumor samples were analyzed separately, in pancreatic NETS, sst1, sst2 and sst5 were overexpressed when compared with control and adjacent tissue; at the same time, a decreased expression of sst3 and sst5TMD4 in tumor samples was observed. In contrast, small bowel and colon tumors tended to progressively increase the expression SST, sst2, sst3, sst5 and sst5TMD4 in adjacent tissue when compared to controls and also in tumors when compared to their respective adjacent tissue and/or normal controls (Supp. Figure 3). Intriguingly, in tumor samples, SST expression was correlated to sst1, sst4 and sst5 and sst5TMD4; in turn, sst5TMD4 was positively correlated to the molecular expression of all evaluated SST system components, while CORT was correlated to sst1. In addition, sst1 expression was correlated with sst4 (Table 3).

Table 3: SST/CORT system components correlations in tumor tissue

	sst1	sst2	sst3	sst4	sst5	sst5TMD4
SST	0,306			0,324	0,288	0,389
CORT	0,344					0,268
sst1				0,288		0,377
sst2			0,902			0,455
sst3						0,460

p<0.05 p<0.01 p<0.001

p value refers to the comparison between SST/CORT system components

Immunohistochemistry (IHC) analysis

Although qPCR is a sensitive method to assess gene expression, we subsequently performed an IHC analysis in a set of selected samples, in order to validate the observed changes at the protein level, and to determine which particular cells are expressing those markers. To this end, we selected sst1, sst2, and sst5 due to their clear overexpression in tumor samples. Specifically, assays performed on FFPE-samples revealed stronger staining in tumor tissue compared to non-tumor adjacent tissue (Fig. 2B). In general, IHC analysis of tumor tissue revealed that somatostatin receptors were present in the vast majority of tumor cells compared with non-tumor adjacent tissue, with different grades of staining (Supp. Figure 4). These data further suggest a dysregulated presence of somatostatin receptors in GEP-NETs samples compared to non-tumor adjacent tissue.

Associations between expression levels of SST system components and clinical-histological characteristics in GEP-NETS patients

Analysis of the demographic, epidemiological and clinical data revealed that patients with tobacco exposure presented GEP-NETs with higher expression of SST and CORT (Figure 3), while in those cases with family history of tumor disease, a lower expression of CORT was observed (Figure 3). Conversely, no correlation was observed with sex, personal history, previous neoplasm history, clinical symptoms or diagnostic techniques.

Importantly, the expression of some SST system components was related to aggressiveness and prognosis of GEP-NETs. Indeed, tumors with lymph node metastasis overexpressed SST while a lower SST and CORT expression was observed in those with liver metastasis. In addition, tumors with free surgical borders overexpressed sst2 and sst3, but tumors with vascular or nerve invasion overexpressed sst5 (Figure 3).

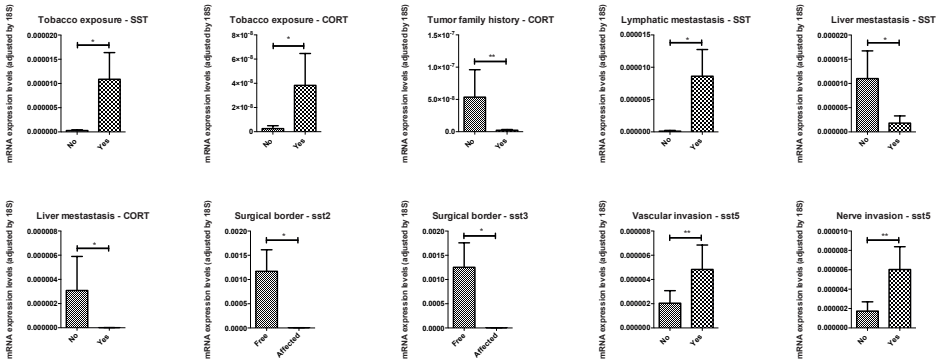


Figure 3: Correlations between epidemiological, clinical, histological and molecular parameters in GEP-NETs. The correlations between epidemiological, clinical, histological and molecular parameters within GEP-NETs samples were assessed by U-Mann Whitney tests. Data represent the mean±SEM. Asterisks (*, p<0.05; **, p<0.01) indicate significant associations.

Effect of SSAs and specific sst agonists on NETs cells survival

To further explore the possible role and potential clinical utility in NET pathophysiology of the two less explored SST receptors, sst1 and sst3, we used two commonly accepted NET model cell lines, BON-1 and QGP-1 cells. Cells were treated with specific sst1 and sst3 agonists and compared with the classic multi-receptor compounds octreotide and pasireotide (Figure 4). This revealed that BON-1 cells decrease their viability rate after an acute treatment (24-h incubation) with sst1 and sst3 analogs (p=0.054 and p<0.05, respectively), whereas no such effect was observed in response to octreotide and pasireotide (Figure 4). In contrast, QGP-1 cell line exhibited an increased survival rate in response to sst1 and sst3 agonists, as well as to octreotide and pasireotide after 24-h of treatment.

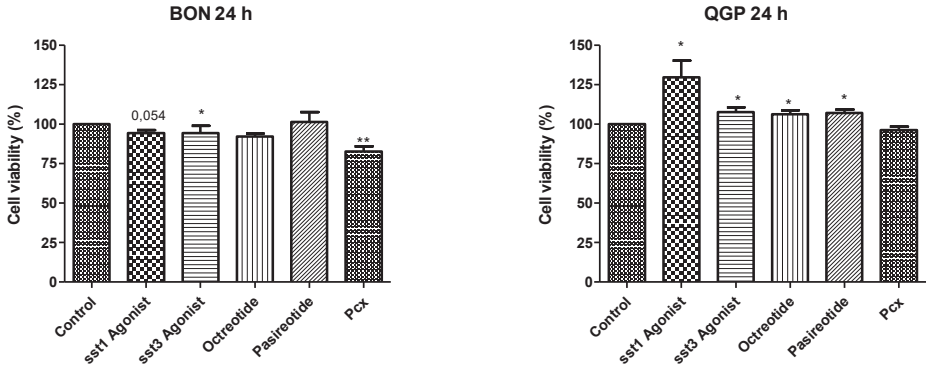


Figure 4: Cell survival rate in BON-1 and QGP-1 cell lines in response to sst stimulators. Cell survival rate was determined by Alamar-blue technique in BON-1 and QGP-1 cells in response to 24h incubation with sst1 agonist, sst3 agonist, octreotide and pasireotide compared to vehicle-treated control. Paclitaxel (Pcx) was used as inhibitory control. Data represent the mean±SEM from at least n=3 independent experiments. Differences were assessed by multiple comparison tests. Asterisks (*, $p<0.05$; **, $p<0.01$) indicate significant differences with control.

DISCUSSION

In the present study, we have systematically evaluated the expression of SST system components (ligands and receptors) in a large series of clinically well-characterized GEP-NETs, including primary tumors of the whole GEP system. Gene expression in tumor tissues was compared with that in the corresponding adjacent non-tumor tissues and in normal control tissues. Some studies [42, 43], including a recent one from our group [20], have explored the presence of certain components of the SST systems in GEP-NETs, but, to the best of our knowledge, this study represents the first comprehensive characterization of the components of this regulatory system in tumor samples, in comparison not only with their corresponding adjacent non-tumor regions but also with normal tissue samples for each analyzed organ. In addition, we thoroughly characterized the demographic, epidemiological, and clinical characteristics of the patients, and the disease progression and prognosis after 2-10 years, and assessed their putative relationship with the expression levels of the components of this SST system. Our results revealed a distinctive expression of most SST system components in tumor and peritumoral tissues compared to normal samples. In addition, specific components of the SST system displayed clinical-histological correlations in tumor tissues, suggesting their potential value as novel markers for GEP-NETs patient management.

The intrinsic variability and heterogeneity that characterize GEP-NETs [44] is further confirmed by the results obtained in this particular cohort of samples, which, first of all, revealed a marked overexpression of CORT, sst1, sst2 and sst5 in tumor tissues compared to

non-tumor adjacent samples. The presence of the different ssts in NETs has been analyzed previously by various groups [15, 24, 42], including ours, in a different cohort of patients [20]. Interestingly, SST system components are expressed not only in tumor tissue but also in non-adjacent tissue, although the expression in tumors is consistently increased compared to the surrounding tissue [20, 24, 45]. These findings, which have been demonstrated by RT-qPCR and IHC, probably relate to their biological regulatory role in secretion and, also, cell proliferation [15], suggesting a dysregulation of these system that could be related with tumor progression. Although the majority of the alterations found in the expression patterns analyzed herein are in agreement with those reported previously (e.g. of sst1 and sst2 overexpression in tumor vs. control tissues) [15, 20, 24, 42], certain changes do not seem to be completely consistent among the different studies, likely owing to obvious differences among the distinct cohorts, but also, to the intrinsic heterogeneity of NETs mentioned above, even when comparable experimental approaches are implemented to characterize tumor expression profiles. In this regard, it is remarkable the difference in the expression of somatostatin receptors in pancreatic NETs when compared to other localizations, and despite the reduced number of samples, our findings underscore the heterogeneity of these tumors, and could be related to lower progression free-survival [46, 47] when compared with other tumors, for example, small bowel NETs [48]. In spite of these differences, and given the emerging relevance of SSAs therapy and peptide receptor radionuclide therapy in the management of well-differentiated GEP-NETs [15, 49, 50], it seems worth emphasizing the high levels of sst2 expression in these tumors observed in our work, which is consistent with previous studies [15, 20, 24, 42], for most currently available SSAs preferentially target this receptor subtype. For this same reason, the sst5 overexpression in tumor tissue observed in our set of samples would also be a relevant marker supporting the potential benefit of SSAs (such as octreotide) as an appropriate treatment for controlling symptoms and tumor growth in well-differentiated GEP-NETs. Furthermore, inasmuch as some tumors are (or become) resistant to SSAs, and some proposed mechanisms suggest defects in sst2 presence, activation, or downstream signaling events [51], it is not unreasonable to propose that the decreased expression of sst2 in less differentiated GEP-NETs observed herein could be related to this phenomenon.

It is also important to note the increased expression of sst2 and sst3 observed in completely resected tumors, which may suggest that the presence of this receptor is associated with the development of well-defined and localized tumors. In fact, it is well known that sst2 can mediates the antiproliferative effects of SST and, as mentioned above, the currently available SSAs have high affinity for this receptor, even its presence and uptake imaging techniques using labeled somatostatin analogues with positron emitting isotopes, especially Gallium-68 being necessary for considering the use of peptide receptor radionuclide therapy in these patients [35, 52-54]. Around 10% of all hepatic metastases are related to a primary NET [55], liver metastatic disease affects between 25 and 90% of NET-patients, and its presence

decreases the 5-year survival about 35-60% [56-58]. Interestingly, in our cohort, liver metastases were not observed in tumors overexpressing SST despite the presence of lymphatic invasion, suggesting a relevant role of SST in tumor behavior. Regarding the expression of other receptors, previous reports have described the presence of the truncated variant of sst5 (sst5TMD4) in breast cancer and poorly differentiated thyroid carcinomas [59, 60]; apparently the balance between this receptor, sst2 and sst5 (full-length) may influence the stage of disease [60]. Similarly, sst5TMD4 has been associated with increased tumor aggressiveness in somatotropinomas and NETS [20, 21] and its dysregulation may also influence the response to SSAs [21, 60]. In the present group of patients, we did not find associations between the truncated isoform and tumor aggressiveness, whereas tumors with vascular and nerve invasion overexpressed sst5, suggesting that a dysregulation in this receptor could be related to aggressive features and the patient outcome.

Indeed, when there is tumor progression or treatment intolerance in response to SSAs, patients require alternative clinical strategies [54, 61, 62], for which novel target treatments [e.g. vascular endothelial growth factor (VEGF) inhibitors and inhibitors of mammalian target of rapamycin (mTOR)] have shown to improve progression-free survival with a low rate of severe adverse events compared with placebo among patients with advanced pancreatic NETs [46, 63]. Unfortunately, despite these new therapeutic options, treatment strategies are still limited and unsatisfactory and, until now, we lack clinical, histological or molecular makers that can satisfactorily predict the evolution of these patients. In this scenario, alternative SST receptors other than sst2, such as sst1 and sst3, could represent suitable options for the development of novel therapeutic strategies. However, expression and functionality of sst1 and sst3 in NETs are hitherto largely unexplored. In our cohort, a significant overexpression of sst1 in tumor tissue was observed, which is consistent with previous reports [20, 64]. On the other hand, although sst3 was not overexpressed in all tumor samples in our cohort, it tended to be overexpressed in less differentiated, more aggressive tumors, and, importantly, it has been found to be overexpressed in other cohorts [20]. These findings suggest the interest of exploring the potential utility of sst1 and sst3 selective agonists in the treatment of NETs. In support of this contention, our present *in vitro* studies demonstrated that treatment with specific sst1 or sst3 agonists was capable to reduce cell survival in BON-1 cells, while, in contrast, SSAs mainly targeting sst2 and/or sst5 (octreotide and pasireotide) were ineffective in this regard. In contrast, QGP-1 cells exhibited an opposite response, wherein treatment with sst1 and sst3 agonists increased cell survival. The reason for these unexpected differences is unknown, but it could be related to the distinct sst1-sst5 expression in tumor cell lines; according to published results, QGP-1 cells have lower mRNA expression levels of all somatostatin receptors, specially sst1 and sst3 [20, 65]. Moreover, similar stimulatory effects on cell survival were observed with other somatostatin analogs (octreotide and pasireotide), in QGP-1 cells but not in BON-1, which suggests that it is a cell type-dependent effect, which could be related to a dysregulation in the

expression, activation or signaling of sst1 and/or sst3, or to their particular interaction with other ssts, in this tumor cell line. These findings further emphasize the potential relevance of NET heterogeneity in the functional response of these tumors to the SSA treatment.

Earlier studies suggested that sst5 could mediate antiproliferative effects when transfected in heterologous cell models [66]. In our cohort, sst5 was overexpressed in tumor tissue compared with adjacent non-tumoral tissue, an abundance that appeared to be associated to aggressive features, for sst5 expression was higher in tumors with vascular and nerve invasion. This suggests that sst5 presence could be related to aggressiveness in NETs, similar to that found in other pathologies [29, 67, 68]. The possible mechanisms underlying this potential association are still to be elucidated. On the other hand, and at variance with previous studies from our group reporting an overexpression of the splicing variant sst5TMD4 in various tumor pathologies, including NETs, in the present set of samples sst5TMD4 was not overexpressed in tumor samples, [20], an observation that could be explained by intrinsic differences in the cohort and would reinforce the idea of the variability and heterogeneity of GEP-NETs [69, 70].

In contrast to the SST receptors, the presence of the ligands SST and CORT in NETs has received less attention in recent years, and given their potential autocrine actions we sought to explore their expression in our cohort. Interestingly, despite its relatively low expression levels, we observed that CORT was clearly overexpressed in tumor samples, and that its levels were higher in patients without family tumor history, suggesting a potential, distinct involvement of this peptide, and its regulation, in NET development and/or progression, which has not been described previously and deserves further investigation. On the other hand, SST was expressed at higher levels but its expression was not altered in tumor vs. not-tumor tissues; however, a closer view to key clinical parameters of GEP-NETs (e.g. invasion capacity) revealed interesting associations, in that tumors with lymph node metastasis overexpressed SST, whereas expression of both SST and CORT was decreased in tumors with liver metastasis, suggesting that a differential expression of SST and CORT could be related to the behavior and aggressiveness in GEP-NETs. In this sense, it has been proposed that CORT synthetic analogs may provide potentially valuable tools as diagnostic/therapeutic tools in SST and ghrelin expressing tumors [10], although their development is still lacking, and should be considered in the future in the field of GEP-NETs.

In summary, this study provides a primary comprehensive mapping of the expression of SST/CORT system component in GEP-NETs, as compared with their respective adjacent non-tumor tissues, and with normal, non-neoplastic tissues. Our results indicate a prominent and widespread overexpression of SST/CORT system components in tumor tissues, wherein they could exert relevant regulatory roles in that the expression levels of some components

are tightly related to the degree of disease, and exhibit associations to key clinical parameters, including invasion and metastasis. Thus, our data provide novel information that might help to better understand NETs pathophysiology and its intrinsic heterogeneity and to identify novel molecular targets with potential prognostic and/or therapeutic value for GEP-NETs patients.

REFERENCES:

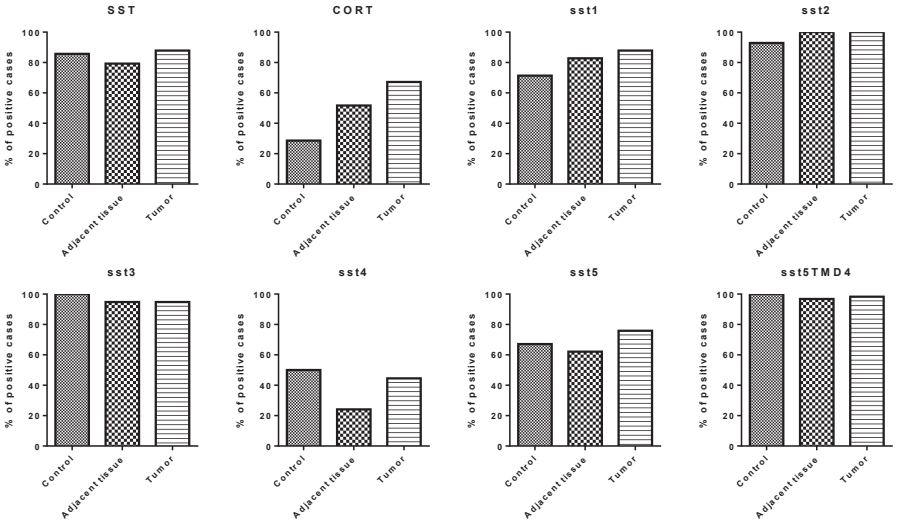
1. Dasari, A., et al., *Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States.* JAMA Oncol, 2017.
2. Tsikitis, V.L., B.C. Wertheim, and M.A. Guerrero, *Trends of incidence and survival of gastrointestinal neuroendocrine tumors in the United States: a seer analysis.* J Cancer, 2012. **3**: p. 292-302.
3. Massironi, S., D. Conte, and R.E. Rossi, *Somatostatin analogues in functioning gastroenteropancreatic neuroendocrine tumours: literature review, clinical recommendations and schedules.* Scand J Gastroenterol, 2015: p. 1-11.
4. Modlin, I.M., K.D. Lye, and M. Kidd, *A 5-decade analysis of 13,715 carcinoid tumors.* Cancer, 2003. **97**(4): p. 934-59.
5. Garcia-Carbonero, R., et al., *SEOM clinical guidelines for the diagnosis and treatment of gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) 2014.* Clin Transl Oncol, 2014. **16**(12): p. 1025-34.
6. Sorbye, H., et al., *Predictive and prognostic factors for treatment and survival in 305 patients with advanced gastrointestinal neuroendocrine carcinoma (WHO G3): the NORDIC NEC study.* Ann Oncol, 2013. **24**(1): p. 152-60.
7. Garcia-Carbonero, R., et al., *Incidence, patterns of care and prognostic factors for outcome of gastroenteropancreatic neuroendocrine tumors (GEP-NETs): results from the National Cancer Registry of Spain (RGETNE).* Ann Oncol, 2010. **21**(9): p. 1794-803.
8. Lawrence, B., et al., *The epidemiology of gastroenteropancreatic neuroendocrine tumors.* Endocrinol Metab Clin North Am, 2011. **40**(1): p. 1-18, vii.
9. Capdevila, J., et al., *Translational research in neuroendocrine tumors: pitfalls and opportunities.* Oncogene, 2017. **36**(14): p. 1899-1907.
10. Volante, M., et al., *Somatostatin, cortistatin and their receptors in tumours.* Mol Cell Endocrinol, 2008. **286**(1-2): p. 219-29.
11. Korner, M., *Specific biology of neuroendocrine tumors: Peptide receptors as molecular targets.* Best Pract Res Clin Endocrinol Metab, 2016. **30**(1): p. 19-31.
12. Pokuri, V.K., M.K. Fong, and R. Iyer, *Octreotide and Lanreotide in Gastroenteropancreatic Neuroendocrine Tumors.* Curr Oncol Rep, 2016. **18**(1): p. 7.
13. Rinke, A., et al., *Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID Study Group.* J Clin Oncol, 2009. **27**(28): p. 4656-63.
14. Caplin, M.E., et al., *Lanreotide in metastatic enteropancreatic neuroendocrine tumors.* N Engl J Med, 2014. **371**(3): p. 224-33.
15. de Herder, W.W., et al., *Somatostatin receptors in gastroentero-pancreatic neuroendocrine tumours.* Endocr Relat Cancer, 2003. **10**(4): p. 451-8.
16. Kaemmerer, D., et al., *Somatostatin receptors in bronchopulmonary neuroendocrine neoplasms: new diagnostic, prognostic, and therapeutic markers.* J Clin Endocrinol Metab, 2015. **100**(3): p. 831-40.
17. Volante, M., F. Bozzalla-Cassione, and M. Papotti, *Somatostatin receptors and their interest in diagnostic pathology.* Endocr Pathol, 2004. **15**(4): p. 275-91.
18. Theodoropoulou, M. and G.K. Stalla, *Somatostatin receptors: from signaling to clinical practice.* Front Neuroendocrinol, 2013. **34**(3): p. 228-52.
19. Lamberts, S.W., W.W. de Herder, and L.J. Hofland, *Somatostatin analogs in the diagnosis and treatment of cancer.* Trends Endocrinol Metab, 2002. **13**(10): p. 451-7.

20. Sampedro-Nunez, M., et al., *Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors.* Oncotarget, 2015.
21. Luque, R.M., et al., *Truncated somatostatin receptor variant sst5TMD4 confers aggressive features (proliferation, invasion and reduced octreotide response) to somatotropinomas.* Cancer Lett, 2015. **359**(2): p. 299-306.
22. Mizutani, G., et al., *Expression of Somatostatin Receptor (SSTR) Subtypes (SSTR-1, 2A, 3, 4 and 5) in Neuroendocrine Tumors Using Real-time RT-PCR Method and Immunohistochemistry.* Acta Histochem Cytochem, 2012. **45**(3): p. 167-76.
23. Hofland, L.J. and S.W. Lamberts, *Somatostatin receptor subtype expression in human tumors.* Ann Oncol, 2001. **12 Suppl 2**: p. S31-6.
24. Herrera-Martinez, A.D., et al., *The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features.* Lung Cancer, 2017. **109**: p. 128-136.
25. Rindi, G., et al., *TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system.* Virchows Arch, 2006. **449**(4): p. 395-401.
26. Duran-Prado, M., et al., *Identification and characterization of two novel truncated but functional isoforms of the somatostatin receptor subtype 5 differentially present in pituitary tumors.* J Clin Endocrinol Metab, 2009. **94**(7): p. 2634-43.
27. Martinez-Fuentes, A.J., et al., *Ghrelin is produced by and directly activates corticotrope cells from adrenocorticotropin-secreting adenomas.* J Clin Endocrinol Metab, 2006. **91**(6): p. 2225-31.
28. Gahete, M.D., et al., *Expression of somatostatin, cortistatin, and their receptors, as well as dopamine receptors, but not of neprilysin, are reduced in the temporal lobe of Alzheimer's Disease patients.* J Alzheimers Dis, 2010. **20**(2): p. 465-475.
29. Taboada, G.F., et al., *Quantitative analysis of somatostatin receptor subtype (SSTR1-5) gene expression levels in somatotropinomas and non-functioning pituitary adenomas.* Eur J Endocrinol, 2007. **156**(1): p. 65-74.
30. Kumar, U., *Expression of somatostatin receptor subtypes (SSTR1-5) in Alzheimer's disease brain: an immunohistochemical analysis.* Neuroscience, 2005. **134**(2): p. 525-38.
31. Breder, C.D., et al., *Differential expression of somatostatin receptor subtypes in brain.* J Neurosci, 1992. **12**(10): p. 3920-34.
32. Luque, R.M., et al., *In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters.* Oncotarget, 2015. **6**(23): p. 19619-33.
33. Kaku, M., et al., *Establishment of a carcinoembryonic antigen-producing cell line from human pancreatic carcinoma.* Gan, 1980. **71**(5): p. 596-601.
34. Uphoff, C.C. and H.G. Drexler, *Detection of mycoplasma contaminations.* Methods Mol Biol, 2013. **946**: p. 1-13.
35. Baldelli, R., et al., *Somatostatin analogs therapy in gastroenteropancreatic neuroendocrine tumors: current aspects and new perspectives.* Front Endocrinol (Lausanne), 2014. **5**: p. 7.
36. Reubi, J.C., et al., *SST3-selective potent peptidic somatostatin receptor antagonists.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13973-8.
37. Cordoba-Chacon, J., et al., *Somatostatin dramatically stimulates growth hormone release from primate somatotrophs acting at low doses via somatostatin receptor 5 and cyclic AMP.* J Neuroendocrinol, 2012. **24**(3): p. 453-63.

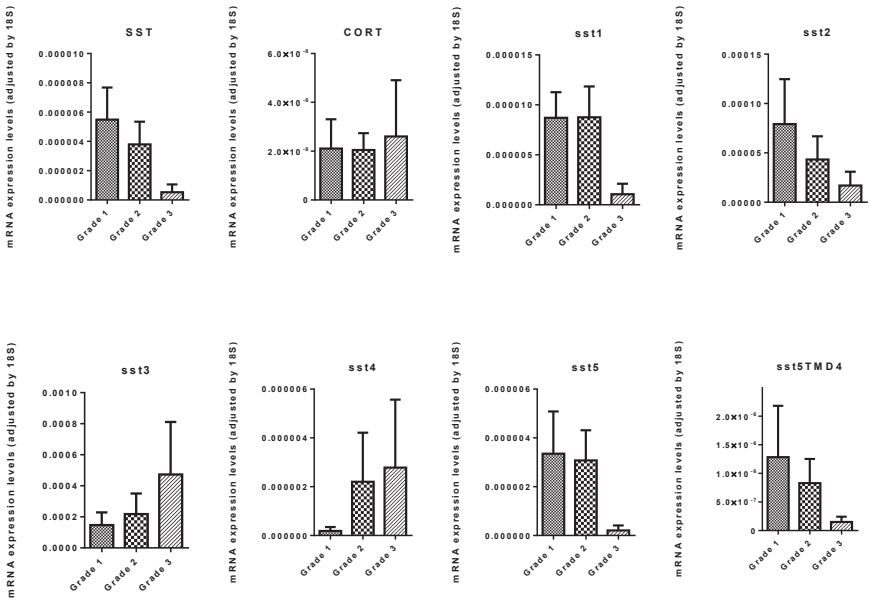
38. van Hoek, M., et al., *Effects of somatostatin analogs on a growth hormone-releasing hormone secreting bronchial carcinoid, in vivo and in vitro studies.* J Clin Endocrinol Metab, 2009. **94**(2): p. 428-33.
39. Hofland, L.J., et al., *The multi-ligand somatostatin analogue SOM230 inhibits ACTH secretion by cultured human corticotroph adenomas via somatostatin receptor type 5.* Eur J Endocrinol, 2005. **152**(4): p. 645-54.
40. Ibanez-Costa, A., et al., *Octreotide and pasireotide (dis)similarly inhibit pituitary tumor cells in vitro.* J Endocrinol, 2016. **231**(2): p. 135-145.
41. Cuny, T., et al., *Somatostatin receptor sst2 gene transfer in human prolactinomas in vitro: impact on sensitivity to dopamine, somatostatin and dopastatin, in the control of prolactin secretion.* Mol Cell Endocrinol, 2012. **355**(1): p. 106-13.
42. Kanakis, G., et al., *Expression of Somatostatin Receptors 1-5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role.* Neuroendocrinology, 2015. **101**(3): p. 211-22.
43. Nasir, A., et al., *Somatostatin receptor profiling in hepatic metastases from small intestinal and pancreatic neuroendocrine neoplasms: immunohistochemical approach with potential clinical utility.* Cancer Control, 2006. **13**(1): p. 52-60.
44. Diez, M., A. Teule, and R. Salazar, *Gastroenteropancreatic neuroendocrine tumors: diagnosis and treatment.* Ann Gastroenterol, 2013. **26**(1): p. 29-36.
45. Li, M., et al., *Characterization of somatostatin receptor expression in human pancreatic cancer using real-time RT-PCR.* J Surg Res, 2004. **119**(2): p. 130-7.
46. Yao, J.C., et al., *Everolimus for advanced pancreatic neuroendocrine tumors.* N Engl J Med, 2011. **364**(6): p. 514-23.
47. Liu, E., P. Marincola, and K. Oberg, *Everolimus in the treatment of patients with advanced pancreatic neuroendocrine tumors: latest findings and interpretations.* Therap Adv Gastroenterol, 2013. **6**(5): p. 412-9.
48. Clift, A.K., et al., *Predicting the survival of patients with small bowel neuroendocrine tumours: comparison of 3 systems.* Endocr Connect, 2017. **6**(2): p. 71-81.
49. Strosberg, J. and L. Kvols, *Antiproliferative effect of somatostatin analogs in gastroenteropancreatic neuroendocrine tumors.* World J Gastroenterol, 2010. **16**(24): p. 2963-70.
50. Schaer, J.C., et al., *Somatostatin receptor subtypes sst1, sst2, sst3 and sst5 expression in human pituitary, gastroentero-pancreatic and mammary tumors: comparison of mRNA analysis with receptor autoradiography.* Int J Cancer, 1997. **70**(5): p. 530-7.
51. Waser, B., et al., *Phosphorylation of sst2 receptors in neuroendocrine tumors after octreotide treatment of patients.* Am J Pathol, 2012. **180**(5): p. 1942-9.
52. Mojtahedi, A., et al., *The value of (68)Ga-DOTATATE PET/CT in diagnosis and management of neuroendocrine tumors compared to current FDA approved imaging modalities: a review of literature.* Am J Nucl Med Mol Imaging, 2014. **4**(5): p. 426-34.
53. Slooter, G.D., et al., *Somatostatin receptor imaging, therapy and new strategies in patients with neuroendocrine tumours.* Br J Surg, 2001. **88**(1): p. 31-40.
54. Bodei, L., G. Pepe, and G. Paganelli, *Peptide receptor radionuclide therapy (PRRT) of neuroendocrine tumors with somatostatin analogues.* Eur Rev Med Pharmacol Sci, 2010. **14**(4): p. 347-51.
55. Benevento, A., et al., *Result of liver resection as treatment for metastases from noncolorectal cancer.* J Surg Oncol, 2000. **74**(1): p. 24-9.
56. Godwin, J.D., 2nd, *Carcinoid tumors. An analysis of 2,837 cases.* Cancer, 1975. **36**(2): p. 560-9.
57. McDermott, E.W., B. Guduric, and M.F. Brennan, *Prognostic variables in patients with gastrointestinal carcinoid tumours.* Br J Surg, 1994. **81**(7): p. 1007-9.

58. Zeitels, J., et al., *Carcinoid tumors: a 37-year experience.* Arch Surg, 1982. **117**(5): p. 732-7.
59. Duran-Prado, M., et al., *The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells.* Oncogene, 2012. **31**(16): p. 2049-61.
60. Mole, D., et al., *The expression of the truncated isoform of somatostatin receptor subtype 5 associates with aggressiveness in medullary thyroid carcinoma cells.* Endocrine, 2015. **50**(2): p. 442-52.
61. Pusceddu, S., et al., *Everolimus treatment for neuroendocrine tumors: latest results and clinical potential.* Ther Adv Med Oncol, 2017. **9**(3): p. 183-188.
62. Faivre, S., et al., *Sunitinib in Pancreatic Neuroendocrine Tumors: Updated Progression-Free Survival and Final Overall Survival From a Phase III Randomized Study.* Ann Oncol, 2016.
63. Raymond, E., et al., *Sunitinib malate for the treatment of pancreatic neuroendocrine tumors.* N Engl J Med, 2011. **364**(6): p. 501-13.
64. Hofland, L.J., et al., *Immunohistochemical detection of somatostatin receptor subtypes sst1 and sst2A in human somatostatin receptor positive tumors.* J Clin Endocrinol Metab, 1999. **84**(2): p. 775-80.
65. van Adrichem, R.C., et al., *Effects of Somatostatin Analogs and Dopamine Agonists on Insulin-Like Growth Factor 2-Induced Insulin Receptor Isoform A Activation by Gastroenteropancreatic Neuroendocrine Tumor Cells.* Neuroendocrinology, 2016. **103**(6): p. 815-25.
66. Cordelier, P., et al., *Characterization of the antiproliferative signal mediated by the somatostatin receptor subtype sst5.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9343-8.
67. Filopanti, M., et al., *Loss of heterozygosity at the SS receptor type 5 locus in human GH- and TSH-secreting pituitary adenomas.* J Endocrinol Invest, 2004. **27**(10): p. 937-42.
68. O'Toole, D., et al., *The analysis of quantitative expression of somatostatin and dopamine receptors in gastro-entero-pancreatic tumours opens new therapeutic strategies.* Eur J Endocrinol, 2006. **155**(6): p. 849-57.
69. Cortez, E., et al., *Functional malignant cell heterogeneity in pancreatic neuroendocrine tumors revealed by targeting of PDGF-DD.* Proc Natl Acad Sci U S A, 2016. **113**(7): p. E864-73.
70. Lapa, C., R.A. Werner, and K. Herrmann, *Visualization of tumor heterogeneity in neuroendocrine tumors by positron emission tomography.* Endocrine, 2016. **51**(3): p. 556-7.

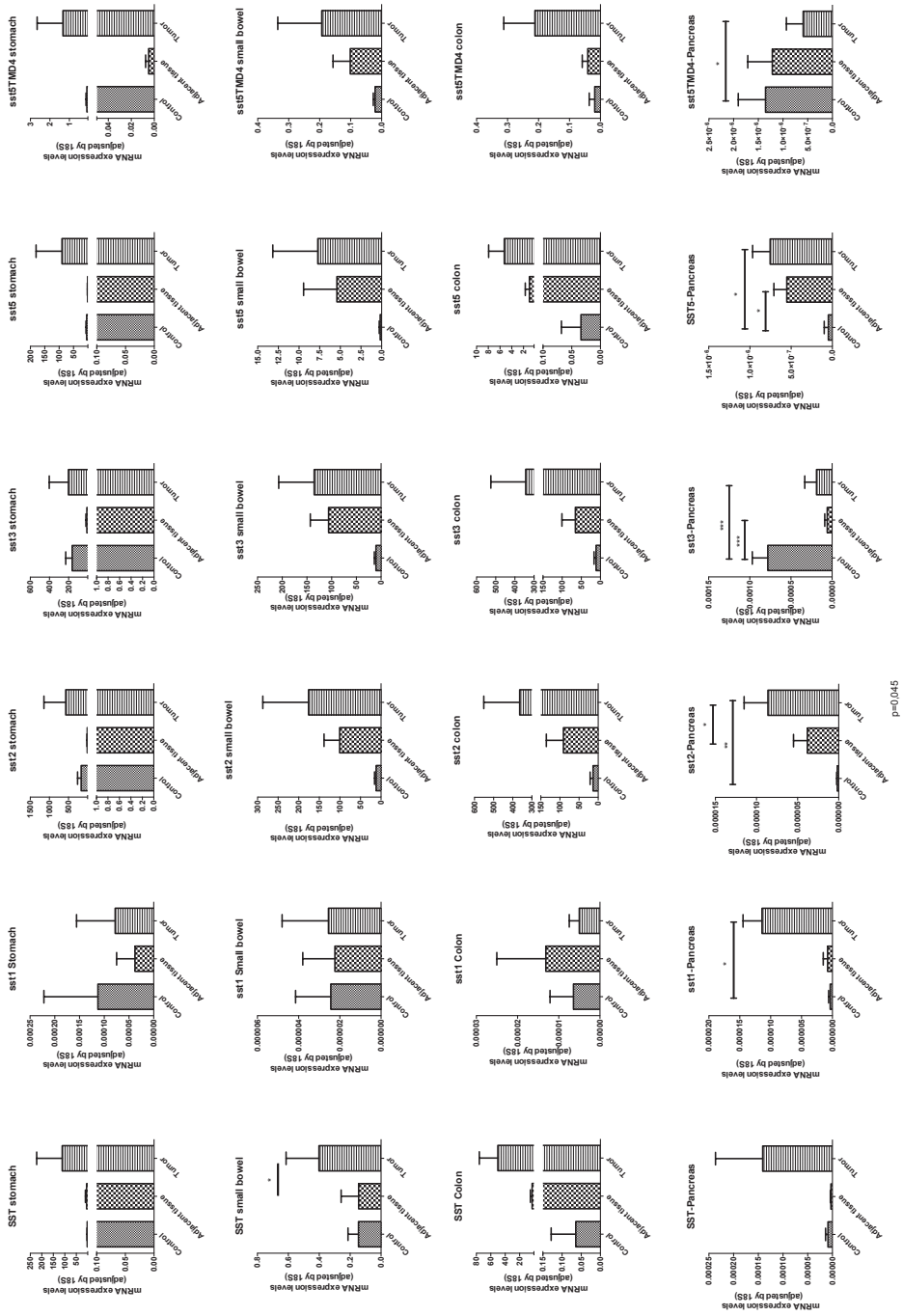
SUPPLEMENTAL DATA



Supplemental Figure 1: Presence of SST/CORT system components in normal tissues, adjacent non-tumor tissue and GEP-NETs. The graphs indicate the percentage of samples (normal control tissue, adjacent non-tumor tissue and tumor tissue) positive for each of the SST/CORT system components.

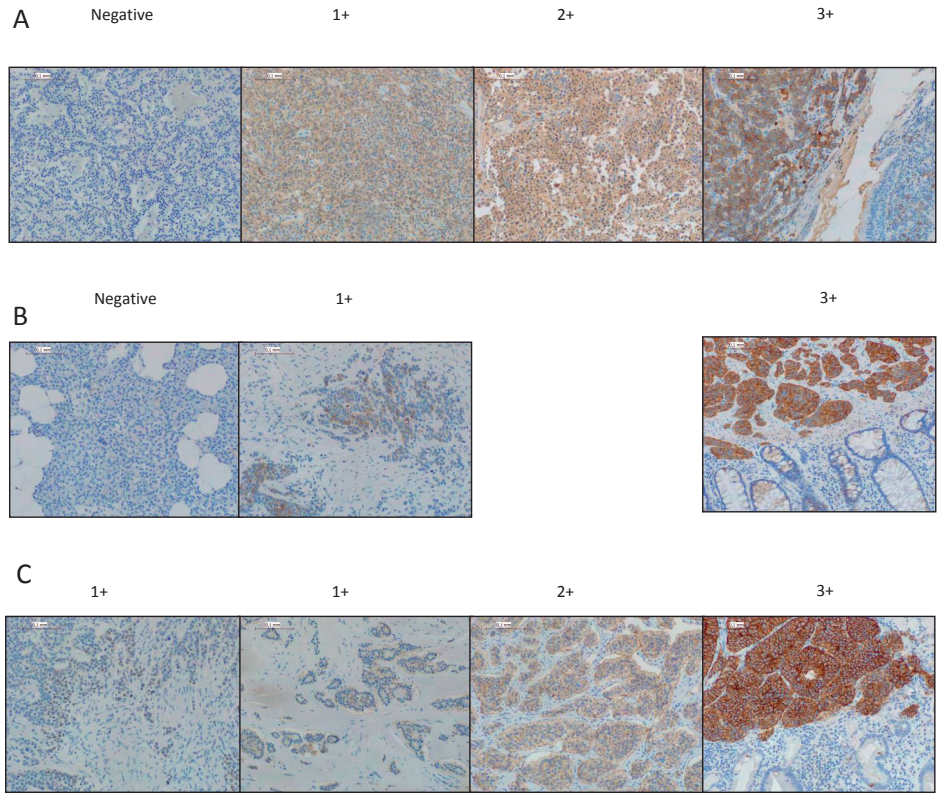


Supplemental Figure 2: Expression of SST/CORT system components in GEP-NETs of different grade. The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in grade 1, 2 and 3 GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean±SEM.



Supplemental Figure 3: Expression of SST system components in normal samples, adjacent non-tumor and tumor tissue of stomach, small bowel, colon and pancreatic NETs. The absolute mRNA

expression of the different components of the SST/CORT system was determined by qPCR in normal control samples, adjacent non-tumor tissue and GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean±SEM. Asterisks (*, p<0.05; **, p<0.01; ***, p<0.001) indicate significant changes by paired analysis between adjacent non-tumor and GEP-NETs and non-paired analysis between normal tissue and adjacent non-tumor or tumor tissues.



Supplemental Figure 4: Presence of sst1, sst2, sst5 by IHC in GEP-NETs. The expression of sst1 **(A)**, sst2 **(B)** and sst5 **(C)** by immunohistochemistry was determined in adjacent non-tumor tissue and NET samples. Samples are classified as negative, 1+, 2+, 3+ staining, which represent absent, low, moderate, and high intensities of the tumor region compared to the adjacent non-tumor tissue.

Chapter 4

Ghrelin O-acyltransferase (GOAT) enzyme as a novel potential biomarker in gastroenteropancreatic neuroendocrine tumors.

Aura D. Herrera-Martínez[1,2], Manuel D. Gahete[1,3], Rafael Sánchez-Sánchez[1,2], Emilia Alors-Perez[1,3], Sergio Pedraza-Arevalo[1,3], Raquel Serrano-Blanch[1,2], Antonio J. Martínez-Fuentes[1,3], Maria A. Gálvez-Moreno[1,2], Justo P. Castaño[1,3], Raúl M. Luque [1,3]

[1] Maimonides Institute for Biomedical Research of Córdoba
[2] Reina Sofi University Hospital
[3] Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Spain

Clinical and Translational Gastroenterology. (2018) 9:196. doi 10.1038/s41424-018-0058-8

ABSTRACT

The association between the presence and alterations of the components of the ghrelin system and the development and progression of neuroendocrine tumors (NETs) is still controversial and remains unclear. Here, we systematically evaluated the expression levels (by quantitative-PCR) of key ghrelin system components of in gastroenteropancreatic (GEP)-NETs, as compared to non-tumor adjacent (NTA; n=42) and normal tissues (NT; n=14). Then, we analyzed their putative associations with clinical-histological characteristics. The results indicate that ghrelin and its receptor GHSR1a are present in a high proportion of normal tissues, while the enzyme ghrelin-O-acyl transferase (GOAT) and the splicing variants In1-ghrelin and GHSR1b were present in a lower proportion of normal tissues. In contrast, all ghrelin system components were present in a high proportion of tumor and NTA tissues. GOAT was significantly overexpressed (by qPCR) in tumor samples compared to NTA, while a trend was found for ghrelin, In1-ghrelin and GHSR1a. In addition, expression of these components displayed significant correlations with key clinical parameters. The marked overexpression of GOAT in tumor samples compared to NTA regions was confirmed by IHC, revealing that this enzyme is particularly overexpressed in gastrointestinal NETs, where it is directly correlated with tumor diameter. These results provide novel information on the presence and potential pathophysiological implications of the ghrelin system components in GEP-NETs, wherein GOAT might represent a novel diagnostic biomarker.

Keywords: GEP-NETs, ghrelin, GOAT, GHSRs, In1-ghrelin, prognosis, invasion

Highlights:

- Key components of ghrelin system are markedly dysregulated in GEP-NETs and associated to key clinical parameters
- Changes in the expression of ghrelin system components are associated with the development and/or progression of GEP-NETs
- These molecular targets, especially GOAT, may represent putative diagnosis and/or prognostic markers in GEP-NETs.

INTRODUCTION

Neuroendocrine tumors (NETs) comprise a heterogeneous family of malignancies with complex clinical behavior and increasing incidence [1-3]. Primary tumor is identified only in 70% of patients [4], while distant metastases are frequently found at diagnosis (27-73%), influencing the overall survival [5-7]. Despite that histological differentiation and Ki67 index are some prognosis factors [4], well-differentiated low-grade tumors may behave aggressively [8]. Unfortunately, surgery is often not applicable since most tumors are diagnosed at advanced stage. For these reasons, the development of novel diagnostic markers has gained scientific and clinical interest [9, 10].

The ghrelin system is involved in the regulation of multiple (patho)-physiological functions, including hormonal secretion, β-cell survival or appetite and gastric motility [11-14]. Ghrelin must undergo a unique modification, consisting of the acylation of the third serine residue, which is catalyzed by the ghrelin-O-acyl-transferase (GOAT) enzyme [14, 15]. Acylated ghrelin (AG) represents the peptide binding and activating its canonical ghrelin receptor, GHSR1a. Interestingly, several ghrelin system variants, resulting from post-transcriptional modifications or alternative splicing, have been identified, including the In1-ghrelin [11, 16] and a truncated receptor GHSR1b, with unknown ligand and function [11, 16, 17].

Alterations in the expression of specific components of this system have been associated with the development/progression of various neoplasms [16, 18-21], including NETs, but the clinical-molecular correlations have not been elucidated [22, 23]. Accordingly, in this study we aimed to: 1) analyze systematically the expression of different components of ghrelin system in gastroenteropancreatic-(GEP-)NETs compared to non-tumor adjacent (NTA) tissue and, most importantly, to normal control tissues by quantitative real-time PCR (qPCR); 2) correlate the expression of these components with clinical/histological characteristics; and 3) perform *in vitro* experiments to elucidate the potential pathophysiological role of GOAT enzyme as a key component particularly altered in our cohort of NET samples, using BON-1 and QGP-1 cell lines.

MATERIALS AND METHODS

Patients and samples

This study was approved by the Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain), was performed according to the Declaration of Helsinki, and patients were treated following national and international clinical practice guidelines. A written informed consent was required before inclusion. Data from 42 patients with GEP-NETs

were collected (demographic and clinical characteristics of the cohort are summarized in Table 1). Additionally, 14 normal control tissues from healthy donors were also included. Patients with hereditary endocrine syndrome were excluded. Clinical records were used to collect full medical history. GEP-NETs were classified according to histopathology features as well-differentiated NETs (G1), moderately differentiated (G2) and poorly differentiated NETs (G3) [24]. Formalin-fixed paraffin-embedded (FFPE) samples were also collected (42 tumor samples, 42 NTA and 14 normal tissues).

Table 1: General characteristics of the patient population

General characteristic		% (n)
Sex		
	Male	52.4% (22)
	Female	47.6% (20)
Age at diagnosis		55.66±17 years
Personal history of other tumors		15.0% (6)
Smoke habit		
	Active	45.0% (9)
	Ex-smoker	20.0% (4)
	No habit	35.0% (7)
Family history of neoplasms		52.9% (9)
Incidental tumor		37.9% (11)
Functionality		43.3% (13)
Mortality rate		18.9% (7)

RNA isolation and reverse-transcription

Total RNA from FFPE samples (n=98) was isolated using the RNeasy-FFPE Kit (Qiagen, Limburg, Netherlands) according to manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC). Total RNA was retrotranscribed to cDNA with the First-Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [25-28]

Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR-Green Master Mix (Thermo Scientific) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Specifically, expression levels (absolute mRNA copy number/50ng of sample) of ghrelin, In1-ghrelin, GOAT-enzyme, GHSR1a and GHSR1b, were measured using previously validated primers [21, 29, 30]. RNA expression was adjusted by *18S* gene expression[28, 31].

Immunohistochemistry (IHC) analysis

IHC analysis of GOAT was implemented in all 42 FFPE samples (tumor and NTA regions) using standard procedures [32]. Optimum antibody concentration (1:300) using a commercially available antibody against human GOAT (AA257-287, Acris-antibodies, Herford, Germany) was selected by performing a series of antibody dilution tests in normal pancreas [33]. Two independent pathologists performed the IHC analysis following a blinded protocol. In the analysis, 0, 1+, 2+, 3+ stand for absent, low, moderate, and high staining intensities of GOAT enzyme in the tumor compared to the NTA region.

Cell culture

In vitro experiments were performed using human NET cell lines BON-1 [34] and QGP-1 [35]. BON-1 cells were cultured in DMEMF12 (Life Technologies, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Madrid, Spain), 1% glutamine (Sigma-Aldrich) and 0.2% antibiotic (Gentamicin/Amphotericin-B; Life Technologies). QGP-1 cells were cultured in RPMI-1640 (Lonza, Basel, Switzerland), supplemented with 10% FBS, 1% glutamine and 0.2% antibiotic. Both cells lines were cultured at 37°C in a 5% CO_2 incubator and monthly checked for mycoplasma contamination by PCR [36].

Cell proliferation assay in response to GOAT inhibitor

The only commercially available GOAT inhibitor (GOATi; GO-CoA-Tat; Ref: 032-37) was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The final concentration (10^{-5}M) was selected based on dose-response experiments performed in prostate cell-lines and on previous reports [37]. Cell proliferation was determined by using Alamar-blue assay (basal, 24h, 48h and 72h) as previously reported [21, 22, 32]. Cells were seeded per quadruplicate and assays were repeated four times. Paclitaxel (PAX; Sigma-Aldrich) was used as control for the inhibition of proliferation [27, 30].

Migration capacity assay

The ability of BON-1 cells to migrate after 24h of treatment was evaluated by wound healing technique [22, 38-40]. Briefly, stable cells were plated at sub-confluence in 6 well plates. The wound was made on confluent cells using a 100 µl sterile pipette tip. Wells were rinsed in PBS and treated for 24 h in FBS-free medium. Wound healing was calculated as the area of a rectangle centered in the picture 24 h after the wound vs. the area of the rectangle just after doing the wound. Three experiments were performed in independent days, in which three random pictures per well along the wound were acquired and, the mean area of these pictures was used for analysis. Images were analyzed using the ImageJ software [41].

Statistical analysis

Paired t-test analysis was used to compare the expression levels between GEP-NETs samples and NTA tissue. Non-paired t-test analysis was used to compare the expression levels between normal tissue and GEP-NETs samples or NTA tissue. U-Mann Whitney tests were used to evaluate clinical-molecular relations within GEP-NETs samples. Chi-squared test was used to compare categorical data. All statistical analyses were performed using SPSS and GraphPad Prism. Data are expressed as mean ± SEM. *p*-values<0.05 were considered statistically significant. In functional experiments, results were expressed as percentage vs. control (vehicle-treated cells). Cell proliferation rate compared to control was assessed by multiple comparison test (Two-way ANOVA followed by Newman-Keuls post-hoc test).

RESULTS

Forty-two patients with GEP-NETs were included. Demographic/clinical features are summarized in Table 1. Specifically, 15 patients presented PNETs and 27 patients presented gastrointestinal (GI)-NETs [52.3% males (22/42); mean age 55.6±17 years]. Tumor characteristics are summarized in Table 2. In our cohort, 43.3% (13/30) were functioning tumors; 63.2% (24/38) had peritumoral invasion [34.3% (12/35) vascular and 35.3% (12/34) neural invasion], 52.4% (22/42) had metastasis at diagnosis [multiple localization in 36.3% (8/22)], 63.2% (24/38) were invasive tumors and the mortality rate reached 18.9% (7/37). Relapsed disease was observed in 36% of patients (13/36). Finally, almost 70% of samples (29/42) were considered as low/intermediate tumors [38.1% (16/42) grade 1 and 31% (13/42) grade 2]. PNETs were statistically larger in size compared to those GI-NETs (4.0±0.47 vs 2.36±0.34 cm respectively; p<0.01).

Histopathological characterization of GEP-NETs and NTA tissue

Primary tumor samples were delimited from the NTA tissues after the evaluation of two experienced pathologists using histology and immunohistochemistry, as previously reported [32].

Expression of components of the ghrelin system in control and GEP-NETs samples

Ghrelin system components were present at variable proportions in normal GEP samples, as determined by qPCR. Ghrelin and its native receptor GHSR1a were expressed in more than 80% of healthy controls (34/42 and 39/42, respectively), while their splicing variants In1-ghrelin and GHSR1b were expressed in about 40% of the samples (17/42 and 19/42, respectively). In contrast, expression of GOAT enzyme was only detected in less than 20% (7/42) of normal samples (Supp. Figure 1). Ghrelin and GHSR1a were also present in a high proportion (more than 60%) of the NTA and tumor samples (32/42 and 29/42,

Table 2: Tumor sample characteristics

Characteristic		% (n)
Tissue samples		
	Primary tumor	42
	Non-tumor adjacent tissue	42
	Normal tissue	14
Primary tumor localization		
	Pancreas	35.7% (15)
	Stomach	7.1% (3)
	Small bowel	31.0% (13)
	Colon and rectum	26.2% (11)
Maximal tumor diameter		2.98±1.86 cm
	Pancreas NETs	4.0±0.47 cm
	Gastrointestinal NETs	2.36±0.34 cm
Necrosis		
	<10%	16.7% (1)
	10-20%	16.7% (1)
	21-30%	50% (3)
	>30%	16.7% (1)
Depth of infiltration (gastrointestinal NETs)		
	Submucosa	4.5% (1)
	Mucosa	4.5% (1)
	Muscular	40.9%(9)
	Serosa	50.0%(11)
Multiple tumors		8.0% (2)
Peri-tumoral tissue invasion		63.2% (24)
Vascular invasion		34.3% (12)
Neural invasion		35.3% (12)
Metastasis		52.4% (22)
Metastasis localization	Liver	9.1% (2)
	Lymphatic nodules	54.5% (12)
	Multiple	36.3% (8)
Grading (WHO 2010 criteria)	Low	38.1% (16)
	Intermediate	31.0% (13)
	High	4.8% (2)
	Unknown	26.2% (11)
Post-surgical treatment		43.2% (16)
Relapsed disease		36.1% (13)
Disease free		55.9% (19)
New surgery requirements		18.2% (6)

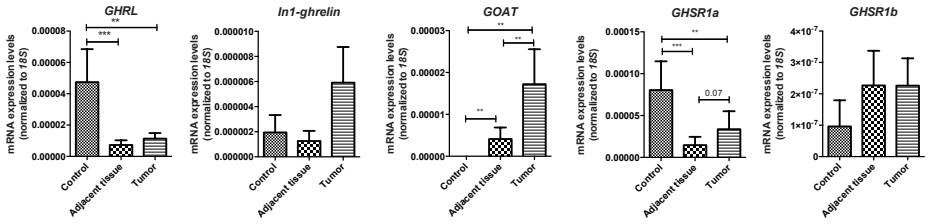


Figure 1: Expression of ghrelin system components in normal GEP, adjacent non-tumor tissue and GEP-NETs. The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in normal GEP controls, adjacent non-tumor tissue and GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean ± SEM. Asterisks (*, p<0.05; **, p<0.01; ***, p<0.001) indicate significant changes by paired analysis between adjacent non-tumor and GEP-NETs and non-paired analysis between normal tissue and adjacent non-tumor or tumor tissues.

respectively); while GOAT enzyme and the splicing variants In1-ghrelin and GHSR1b were present in more than 40% of the samples ((25/42, 21/42 and 17/42, respectively; Supp. Figure 1). Of note, ghrelin expression levels were decreased in NTA and tumor tissue compared with normal samples, with a slightly but not significantly increased expression in tumor compared with NTA tissue (Figure 1). A similar observation was found for GHSR1a expression, while GOAT enzyme was clearly overexpressed in tumor tissues compared with NTA regions and normal tissues, wherein it was virtually absent (Figure 1). Finally, In1-ghrelin was more expressed in tumor tissues than in control samples but these differences were not statistically significant, while no significant changes were found in the case of the splicing variant GHSR1b (Figure 1).

In terms of tumor grade, no significant differences in the expression of any of the ghrelin system components analyzed were found between differentiated (G1/G2) and non-differentiated (G3) GEP-NET (Supp. Figure 2). However, we found that the expression of GOAT enzyme and GHSR1a in GI-NETs was markedly higher than in PNETs, while the expression of ghrelin was lower in GI-NETs compared to PNETs (Supp. Figure 3). Additionally, ghrelin expression levels correlated with those of In1-ghrelin (R^2=0.532; p<0.01) and GOAT (R^2=0.422; p<0.05) in tumor samples, while the expression of GHSR1a was correlated with GHSR1b (R^2=0.444; p<0.05).

Immunohistochemistry analysis of the presence of GOAT enzyme

Based on the marked overexpression of GOAT enzyme, as well as on previous reports [16, 18], we also sought to analyze its presence at the protein level. IHC analysis of tumor tissue revealed that GOAT enzyme was present in the vast majority of tumor cells compared with NTA tissue (Figure 2A), with different grades of staining. Indeed, in our cohort, 86% of the tumor samples (36/42) evaluated were positive for the presence of GOAT enzyme by IHC (Figure 2B), wherein 40% of the tumor cases (17/42) presented a strong staining (2+ or 3+)

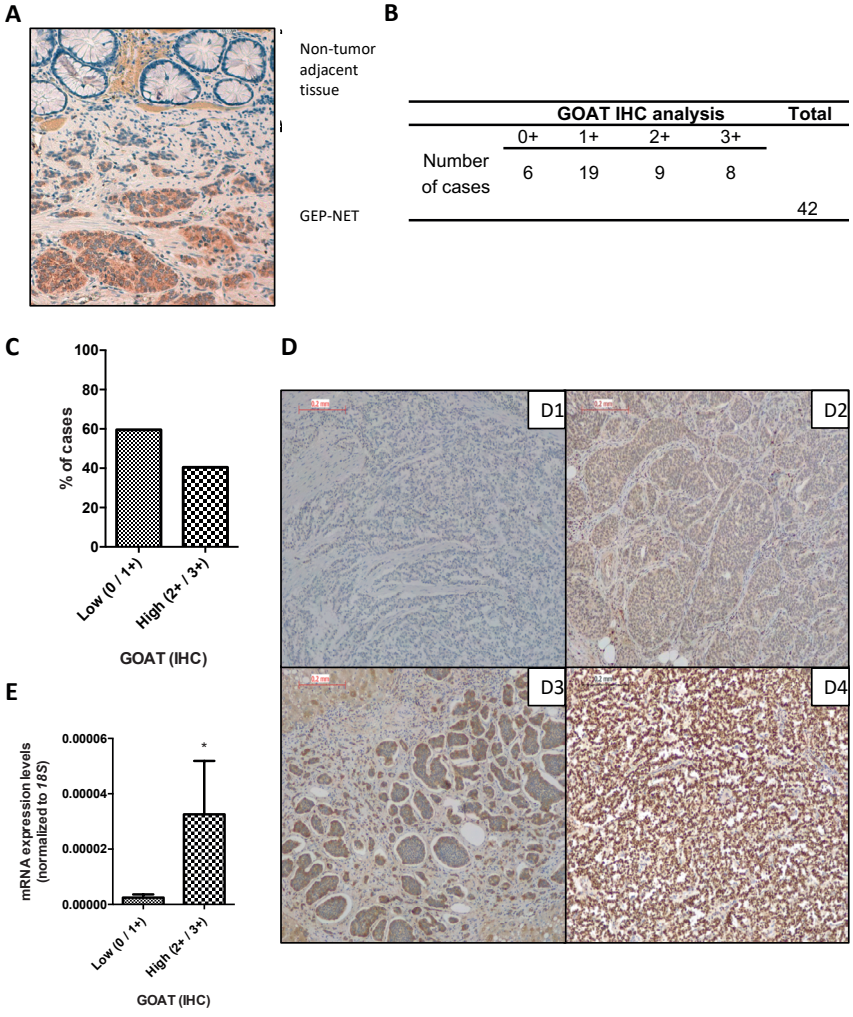


Figure 2: Immunohistochemical analysis of GOAT enzyme in adjacent non-tumor tissue and GEP-NETs. The presence of GOAT by immunohistochemistry using a specific antibody was determined in a subset of samples, which included tumor and non-tumor regions from patients diagnosed with GEP-NETs. **A)** Representative images of the IHC analysis of GOAT enzyme in a GEP-NET sample compared with the non-tumor adjacent tissue. **B)** Absolute number of cases according to the intensity of GOAT IHC staining (0, 1+, 2+, 3+). **C)** The graph indicates the percentage of tumor samples according to the intensity of GOAT expression by IHC, 0 and 1+ have been grouped as low expression while 2+ and 3+ have been grouped as high intensity by IHC. **D)** Representative images of different GOAT staining in GEP-NETs. In the analysis, 0, 1+, 2+, 3+ stand for absent, low, moderate, and high intensities of the tumor region staining compared to the adjacent region with non-tumor tissue (3C1, 3C2, 3C3, 3C4 respectively). This analysis revealed that GOAT was present in the vast majority of tumor cells compared with non-tumor adjacent tissue, with different grades of staining. **E)** Correlation between the absolute mRNA expression of GOAT determined by qPCR in GEP-NETs samples (values are adjusted by 18S expression) and the intensity of GOAT staining.

for GOAT compared to NTA tissue (Figure 2C and 2D). Of note, mRNA expression levels in tumors samples correlated with GOAT expression by IHC (Figure 2E). Additionally, strong staining (2+ or 3+) for GOAT was correlated to increased age at diagnosis (62.5±4 years) compared to those tumors with absent or lower staining (1+; 51±2 years; $p>0.05$).

Correlations between the expression levels of ghrelin system components and clinical-histological characteristics in GEP-NETs

Epidemiological data revealed that patients with tobacco exposure exhibited higher expression of ghrelin and In1-ghrelin (Figure 3A). Moreover, patients with family history of tumor disease had a lower expression of ghrelin (Figure 3B). Conversely, sex, personal history, previous neoplasm history, clinical symptoms, or other histological parameters (vascular/peritumoral invasion, lymph node metastasis) were not associated with the expression of any of the components of the ghrelin system.

Expression of some ghrelin system components was also associated to tumor characteristics, invasion capacity and prognosis in GEP-NETs. Specifically, functioning tumors presented higher levels of GHSR1a (Figure 3C), while lower expression levels of this receptor were associated to the presence of affected surgical borders and mortality (Figure 3C). Tumors with necrosis had lower GOAT mRNA levels and those with liver metastasis had decreased expression levels of In1-ghrelin (Figure 3D). Interestingly, functionality was also associated with increased expression of GHSR1b (Figure 3E). Finally, tumor diameter was directly correlated to GOAT expression ($R=0.33$; $p<0.05$). Remarkably, no further associations were found between expression levels of ghrelin system components and clinical/histological characteristics when considering separately PNETs and GI-NETs (data not shown)

In vitro analysis of the role of GOAT in PNETs cell lines

We decided to further investigate the pathophysiological role of GOAT enzyme using the only available GOATi in PNETs cell lines. However, GOATi did not affect cell proliferation in BON-1 and QGP-1 cells (Figure 4A) or the migration capacity of BON-1 cells (Figure 4B).

DISCUSSION

This study aimed at evaluating systematically the expression of various components of the ghrelin system in an ample series of clinically well-characterized GEP-NETs, and to compare these expression levels with those in the corresponding adjacent non-tumor tissues and in normal control tissues. Previous studies have reported certain components of the ghrelin system in GEP-NETs [22, 42-44]; however, to our knowledge, this is the first study that comprehensively characterizes these components in tumor samples compared to their cor-

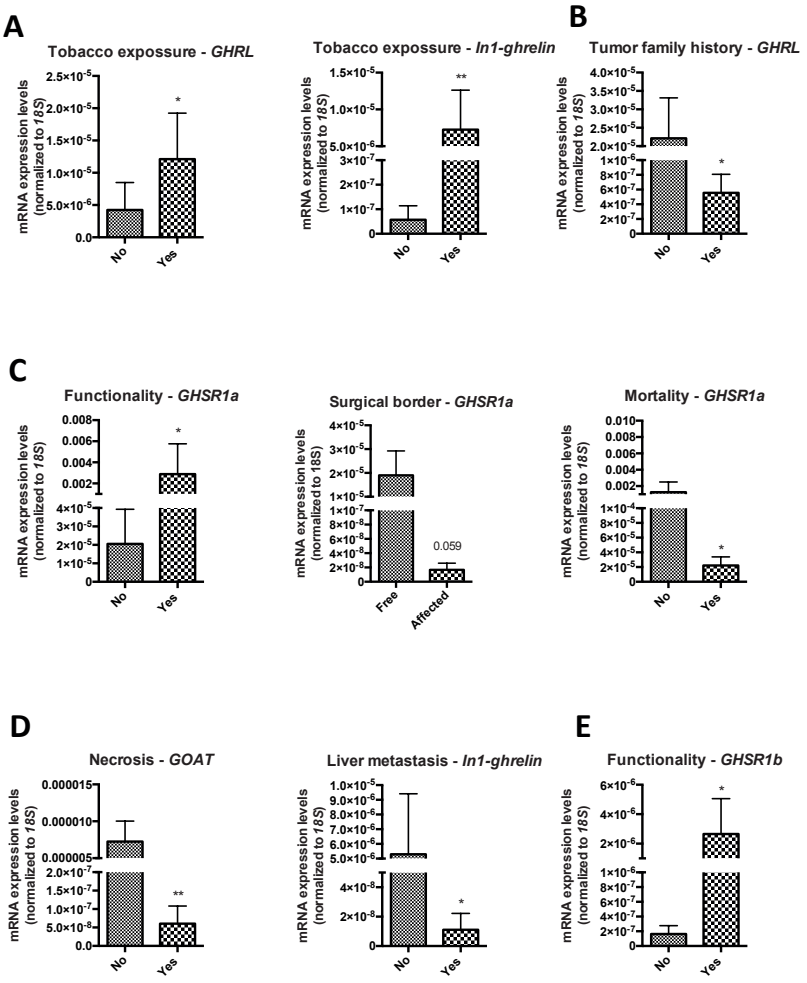


Figure 3: Correlations between epidemiological, clinical, histological and molecular parameters in GEP-NETs. The correlations between epidemiological, clinical, histological and molecular parameters within GEP-NET samples were assessed by U-Mann Whitney tests. Asterisks indicate significant associations (*, p<0.05; **, p<0.01; ***, p<0.001).

responding adjacent non-tumor regions, as well as with normal tissue samples. Moreover, we analyzed the demographic, epidemiological, and clinical characteristics as well as the disease progression and prognosis after 2–10 years of the patients with GEP-NETs. Overall, our results revealed that most of the components of the ghrelin system exhibit a distinctive expression in tumor and peritumoral tissues compared to normal tissue samples. Indeed, specific components of the ghrelin system, and especially GOAT, displayed remarkable alterations and clinical-histological correlations in tumor tissues, suggesting their potential value as novel biomarkers in GEP-NETs.

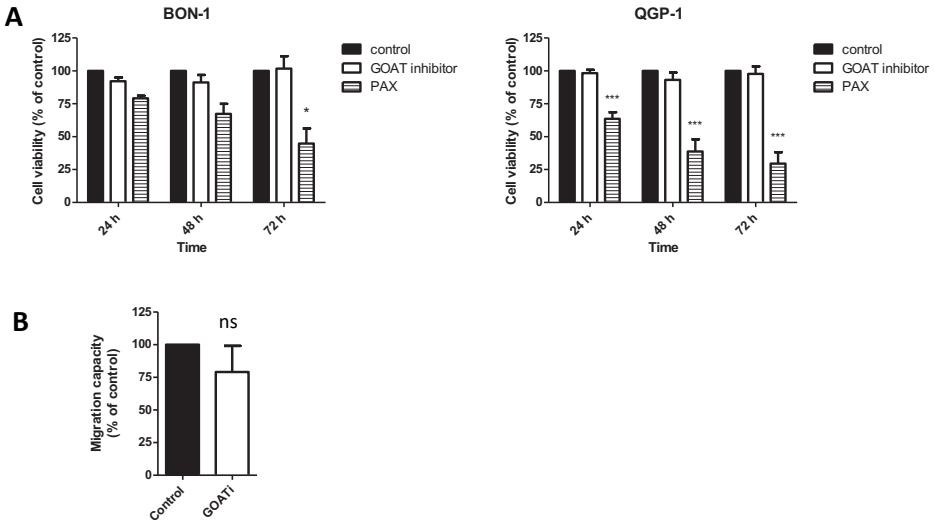


Figure 4: *In vitro* analysis of the consequences of GOAT inhibitor (GOATi) treatment in NET cell lines. A) Cell proliferation rate in BON-1 and QGP-1 cell lines after 24h, 48h and 72h of GOATi treatment determined by Alamar-blue assay. Paclitaxel (PAX) was used as inhibitory control in proliferation assays. B) Cell migration rate in BON-1 after 24h of treatment with GOAT inhibitor by wound-healing assay. Cell proliferation rate compared to control was assessed by multiple comparison tests while migration was assessed by U-Mann Whitney test. Values represent the mean ± SEM of at least three individual experiments. Asterisks indicate significant differences (*,$p<0.05$; ***,$p<0.001$) compared with control (set at a 100%). Legend: ns means non-significant.

Also in our cohort, GEP-NETs exhibited a substantial molecular heterogeneity and variability [22, 32, 45]. Our results are consistent with previous reports showing that different components of the ghrelin system are present in tumor and non-adjacent tissues, and that, some of these components can be overexpressed in tumor samples compared to the surrounding tissue [22, 42-44]. Differences among these studies may be related to the differences among patient cohorts.

Ghrelin system regulates key bodily functions, such as hormonal secretion and cell proliferation, in both normal and tumor cells [11-13, 46, 47]. In this context, our and other studies support the notion that the dysregulation of ghrelin system components observed in NETs could be pathologically relevant and may participate in tumor progression. The diverse localization and morphology of ghrelin-producing cells in the GI tract, and their implications on metabolic/endocrine functions, might suggest a role of this component in the regulation of GEP-NETs patho-physiology [12, 13, 42], and could also explain the ample molecular heterogeneity found herein in the expression of ghrelin in different normal control tissues. Moreover, it could also be related to the overall overexpression of ghrelin in normal tissues compared to tumor samples. Although expression of canonical ghrelin has been described

in various tumor types, its potential role in cancer is still controversial [48, 49]. Ghrelin has been described in NETs using immunohistochemistry and qPCR [22, 25, 42, 43], in our cohort ghrelin was expressed in NET samples, albeit in substantially lower amounts than in normal tissues. In addition, in our cohort ghrelin expression levels were higher in PNETs than in GI-NETs, which is consistent with previous evidence [43] but differed from other reports [44]. At variance with previous studies that did not find any clinical correlation between ghrelin expression and clinical features [22, 42], we observed here that ghrelin expression was higher in patients without tumor family history. Similarly, the *in vitro* effects of ghrelin on cell proliferation are also controversial [48, 50-56] and some studies have reported an association between ghrelin and poor survival in renal cell carcinoma patients [57, 58]. Altogether, these data reinforce the notion that NETs are highly heterogeneous tumors, wherein the particular ghrelin expression profile and its clinical implications may depend on the type of tumor and the particular cohort of patients analyzed.

Expression of the canonical ghrelin receptor GHSR1a has been described in tumors including NETs [22, 25, 48]. Here, GHSR1a expression was highly variable in normal control samples, but tended to be overexpressed in tumor samples compared to adjacent non-tumor tissue, which is consistent with our previous study in a different cohort [22]. The relation between GHSR1a, functionality and mortality invites to explore further the potential relationship of this receptor with tumorigenesis, and its putative value as a molecular prognostic marker in NETs.

The pathophysiological implications of the ghrelin system have been recently expanded with the discovery of new molecular components [11, 16, 17, 29], which have been found to be overexpressed in several tumors [16, 22, 27] and associated to relevant clinical parameters [22]. Herein we found comparable tendencies in the expression of these variants; however, these differences did not reach statistical significance. Nevertheless, in the present cohort, more than 40% of tumor samples presented detectable levels of In1-ghrelin and GHSR1b, while in the previous study, more than 80% of the tumor samples exhibited detectable levels [22]. These differences could likely reduce the statistical power of the comparisons and correlations, and, again, would illustrate the elevated heterogeneity of NETs.

The most novel and relevant finding of this study is the marked overexpression of GOAT in NET samples. Whereas the expression of this enzyme was almost absent in control tissues, it was present in adjacent non-tumor tissue and notably overexpressed in tumor tissues. These, together with previous results showing a similar, remarkable overexpression of GOAT in breast and pituitary tumors [11, 21] provide suggestive evidence for a striking dysregulation of this enzyme in endocrine-related tumors. The expression levels GOAT does not always correlate with those of ghrelin, whereas they do parallel more consistently the expression levels of In1-ghrelin, suggesting the existence of additional targets for GOAT enzyme [11]. In NETs, GOAT levels have been correlated with those of In1-ghrelin, and associated with worse outcome [22], these findings were not reproducible in our cohort, which may be

explained by the tumor heterogeneity and the limited number of tumor samples. Despite this, in the present study, GOAT expression is associated to larger tumors, especially in GI-NETs, reinforcing the notion of a possible association between the dysregulation of this enzyme and the pathophysiology of NETs. This is the first study that demonstrates an intense overexpression of GOAT enzyme by IHC in GEP-NETs tissues compared to non-tumor adjacent tissues; however, its functional implications should be precisely defined. Here, a GOAT inhibitor administered on two NET cell lines, BON-1 and QGP-1, did not show relevant changes in cell proliferation or migration *in vitro*. Thus, future studies should explore this further, using novel inhibitors or other inhibiting/silencing approaches.

Notwithstanding this, our current and previous [22] studies provide compelling evidence that certain components of the ghrelin system, and specially GOAT enzyme, are clearly overexpressed in NETs, suggesting their potential value as diagnostic and/or prognostic biomarkers for this pathology. In support of the present finding in NETs, GOAT has been also recently reported as non-invasive plasma biomarker in prostate cancer [18]. Additionally, the association between GHSR1a and GHSR1b with the functionally of these tumors and the mortality of these patients further supports this notion and emphasizes the importance of exploring the modulation of this receptor for improving patient outcome. Therefore, although it is difficult to predict the specific clinical impact of these findings, taken together, all these results invite to analyze in more detail the putative utility of GOAT overexpression as a diagnostic biomarker in NETs.

In summary, we present the first systematic characterization of the components of the ghrelin system, including splicing variants, in GEP-NETs tissues in comparison with their adjacent non-tumor regions, and also with normal tissue samples. Our results demonstrate that key components of this system are markedly dysregulated in GEP-NETs and associated to key clinical parameters, suggesting the interest of further studying these molecular targets, especially GOAT, as putative diagnosis and/or prognostic markers in GEP-NETs.

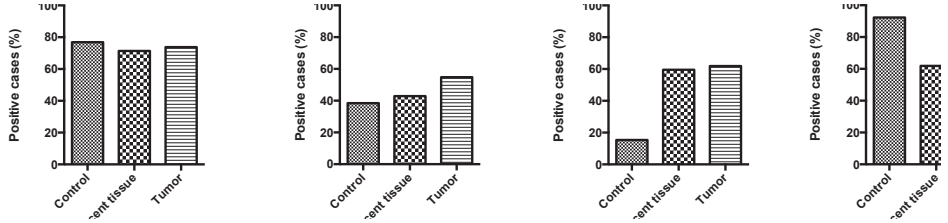
REFERENCES:

1. Diez, M., A. Teule, and R. Salazar, *Gastroenteropancreatic neuroendocrine tumors: diagnosis and treatment.* Ann Gastroenterol, 2013. **26**(1): p. 29-36.
2. Dasari, A., et al., *Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States.* JAMA Oncol, 2017.
3. Oberg, K., et al., *Molecular pathogenesis of neuroendocrine tumors: implications for current and future therapeutic approaches.* Clin Cancer Res, 2013. **19**(11): p. 2842-9.
4. Sorbye, H., et al., *Predictive and prognostic factors for treatment and survival in 305 patients with advanced gastrointestinal neuroendocrine carcinoma (WHO G3): the NORDIC NEC study.* Ann Oncol, 2013. **24**(1): p. 152-60.
5. Garcia-Carbonero, R., et al., *Incidence, patterns of care and prognostic factors for outcome of gastroenteropancreatic neuroendocrine tumors (GEP-NETs): results from the National Cancer Registry of Spain (RGETNE).* Ann Oncol, 2010. **21**(9): p. 1794-803.
6. Garcia-Carbonero, R., et al., *SEOM clinical guidelines for the diagnosis and treatment of gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) 2014.* Clin Transl Oncol, 2014. **16**(12): p. 1025-34.
7. Lawrence, B., et al., *The epidemiology of gastroenteropancreatic neuroendocrine tumors.* Endocrinol Metab Clin North Am, 2011. **40**(1): p. 1-18, vii.
8. Massironi, S., D. Conte, and R.E. Rossi, *Somatostatin analogues in functioning gastroenteropancreatic neuroendocrine tumours: literature review, clinical recommendations and schedules.* Scand J Gastroenterol, 2015: p. 1-11.
9. Castano, J.P., et al., *Gastrointestinal neuroendocrine tumors (NETs): new diagnostic and therapeutic challenges.* Cancer Metastasis Rev, 2014. **33**(1): p. 353-9.
10. Capdevila, J., et al., *Translational research in neuroendocrine tumors: pitfalls and opportunities.* Oncogene, 2017. **36**(14): p. 1899-1907.
11. Gahete, M.D., et al., *Ghrelin gene products, receptors, and GOAT enzyme: biological and pathophysiological insight.* J Endocrinol, 2014. **220**(1): p. R1-24.
12. Chopin, L.K., et al., *The ghrelin axis--does it have an appetite for cancer progression?* Endocr Rev, 2012. **33**(6): p. 849-91.
13. Kojima, M., et al., *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature, 1999. **402**(6762): p. 656-60.
14. Muller, T.D., et al., *Ghrelin.* Mol Metab, 2015. **4**(6): p. 437-60.
15. Nishi, Y., et al., *Structures and molecular forms of the ghrelin-family peptides.* Peptides, 2011. **32**(11): p. 2175-82.
16. Gahete, M.D., et al., *A novel human ghrelin variant (In1-ghrelin) and ghrelin-O-acyltransferase are overexpressed in breast cancer: potential pathophysiological relevance.* PLoS One, 2011. **6**(8): p. e23302.
17. Leung, P.K., et al., *The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor.* Cell Signal, 2007. **19**(5): p. 1011-22.
18. Hormaechea-Agulla, D., et al., *Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer, and its levels are associated with patient's metabolic status: Potential value as a non-invasive biomarker.* Cancer Lett, 2016. **383**(1): p. 125-134.
19. Pokuri, V.K., M.K. Fong, and R. Iyer, *Octreotide and Lanreotide in Gastroenteropancreatic Neuroendocrine Tumors.* Curr Oncol Rep, 2016. **18**(1): p. 7.

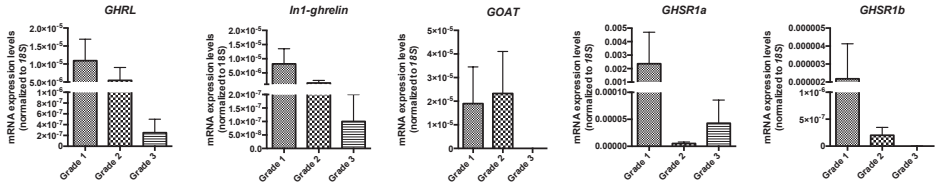
20. Ucan, B., et al., *Serum ghrelin levels in papillary thyroid carcinoma.* Arch Endocrinol Metab, 2017. **61**(5): p. 464-469.
21. Ibanez-Costa, A., et al., *In1-ghrelin splicing variant is overexpressed in pituitary adenomas and increases their aggressive features.* Sci Rep, 2015. **5**: p. 8714.
22. Luque, R.M., et al., *In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters.* Oncotarget, 2015. **6**(23): p. 19619-33.
23. Corbetta, S., et al., *Circulating ghrelin levels in patients with pancreatic and gastrointestinal neuroendocrine tumors: identification of one pancreatic ghrelinoma.* J Clin Endocrinol Metab, 2003. **88**(7): p. 3117-20.
24. Rindi, G., et al., *TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system.* Virchows Arch, 2006. **449**(4): p. 395-401.
25. Herrera-Martinez, A.D., et al., *The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features.* Lung Cancer, 2017. **109**: p. 128-136.
26. Hormaechea-Agulla, D., et al., *The oncogenic role of the spliced somatostatin receptor sst5TMD4 variant in prostate cancer.* FASEB J, 2017.
27. Hormaechea-Agulla, D., et al., *The oncogenic role of the In1-ghrelin splicing variant in prostate cancer aggressiveness.* Mol Cancer, 2017. **16**(1): p. 146.
28. Herrera-Martinez, A.D., et al., *Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors.* Endocrine, 2018. **59**(2): p. 426-437.
29. Gahete, M.D., et al., *Role of ghrelin system in neuroprotection and cognitive functions: implications in Alzheimer's disease.* Peptides, 2011. **32**(11): p. 2225-8.
30. Rincon-Fernandez, D., et al., *In1-ghrelin splicing variant is associated with reduced disease-free survival of breast cancer patients and increases malignancy of breast cancer cells lines.* Carcinogenesis, 2017.
31. Rubie, C., et al., *Housekeeping gene variability in normal and cancerous colorectal, pancreatic, esophageal, gastric and hepatic tissues.* Mol Cell Probes, 2005. **19**(2): p. 101-9.
32. Herrera-Martinez, A.D., et al., *Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors.* Endocrine, 2017.
33. Lim, C.T., et al., *The expression of ghrelin O-acyltransferase (GOAT) in human tissues.* Endocr J, 2011. **58**(8): p. 707-10.
34. Evers, B.M., et al., *Establishment and characterization of a human carcinoid in nude mice and effect of various agents on tumor growth.* Gastroenterology, 1991. **101**(2): p. 303-11.
35. Kaku, M., et al., *Establishment of a carcinoembryonic antigen-producing cell line from human pancreatic carcinoma.* Gan, 1980. **71**(5): p. 596-601.
36. Uphoff, C.C. and H.G. Drexler, *Detection of mycoplasma contaminations.* Methods Mol Biol, 2005. **290**: p. 13-23.
37. Barnett, B.P., et al., *Glucose and weight control in mice with a designed ghrelin O-acyltransferase inhibitor.* Science, 2010. **330**(6011): p. 1689-92.
38. Luque, R.M., et al., *Evidence that endogenous SST inhibits ACTH and ghrelin expression by independent pathways.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E395-403.
39. Duran-Prado, M., et al., *The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells.* Oncogene, 2012. **31**(16): p. 2049-61.

40. Gahete, M.D., et al., *The truncated somatostatin receptor sst5TMD4 stimulates the angiogenic process and is associated to lymphatic metastasis and disease-free survival in breast cancer patients.* Oncotarget, 2016. **7**(37): p. 60110-60122.
41. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis.* Nat Methods, 2012. **9**(7): p. 671-5.
42. Papotti, M., et al., *Ghrelin-producing endocrine tumors of the stomach and intestine.* J Clin Endocrinol Metab, 2001. **86**(10): p. 5052-9.
43. Rindi, G., et al., *Ghrelin expression in gut endocrine growths.* Histochem Cell Biol, 2002. **117**(6): p. 521-5.
44. Volante, M., et al., *Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors.* J Clin Endocrinol Metab, 2002. **87**(3): p. 1300-8.
45. Cives, M., H.P. Soares, and J. Strosberg, *Will clinical heterogeneity of neuroendocrine tumors impact their management in the future? Lessons from recent trials.* Curr Opin Oncol, 2016. **28**(4): p. 359-66.
46. Rotondo, F., et al., *Ghrelin immunoexpression in the human hypophysis.* Appl Immunohistochem Mol Morphol, 2012. **20**(1): p. 77-81.
47. Yanagi, S., et al., *The Homeostatic Force of Ghrelin.* Cell Metab, 2018. **27**(4): p. 786-804.
48. Lin, T.C. and M. Hsiao, *Ghrelin and cancer progression.* Biochim Biophys Acta, 2017. **1868**(1): p. 51-57.
49. Sever, S., D.L. White, and J.M. Garcia, *Is there an effect of ghrelin/ghrelin analogs on cancer? A systematic review.* Endocr Relat Cancer, 2016. **23**(9): p. R393-409.
50. Kraus, D., et al., *Ghrelin promotes oral tumor cell proliferation by modifying GLUT1 expression.* Cell Mol Life Sci, 2016. **73**(6): p. 1287-99.
51. Lien, G.S., et al., *Ghrelin induces colon cancer cell proliferation through the GHS-R, Ras, PI3K, Akt, and mTOR signaling pathways.* Eur J Pharmacol, 2016. **776**: p. 124-31.
52. Cassoni, P., et al., *Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines.* J Clin Endocrinol Metab, 2001. **86**(4): p. 1738-45.
53. Cassoni, P., et al., *Ghrelin and cortistatin in lung cancer: expression of peptides and related receptors in human primary tumors and in vitro effect on the H345 small cell carcinoma cell line.* J Endocrinol Invest, 2006. **29**(9): p. 781-90.
54. Raghay, K., et al., *Ghrelin localization in rat and human thyroid and parathyroid glands and tumours.* Histochem Cell Biol, 2006. **125**(3): p. 239-46.
55. Diaz-Lezama, N., et al., *Ghrelin inhibits proliferation and increases T-type Ca2+ channel expression in PC-3 human prostate carcinoma cells.* Biochem Biophys Res Commun, 2010. **403**(1): p. 24-9.
56. Bai, R.X., et al., *Ghrelin attenuates the growth of HO-8910 ovarian cancer cells through the ERK pathway.* Braz J Med Biol Res, 2016. **49**(3).
57. Aguirre-Gamboa, R., et al., *SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis.* PLoS One, 2013. **8**(9): p. e74250.
58. Lin, T.C., et al., *Ghrelin promotes renal cell carcinoma metastasis via Snail activation and is associated with poor prognosis.* J Pathol, 2015. **237**(1): p. 50-61.

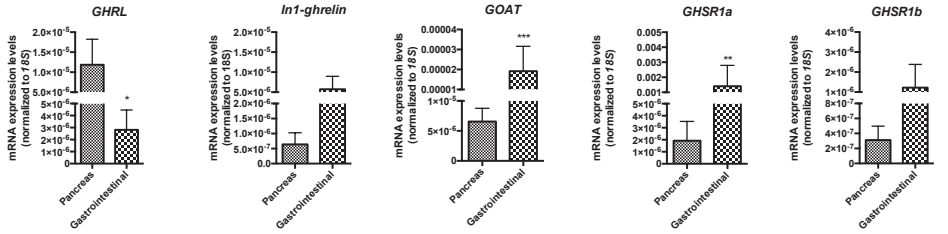
SUPPLEMENTAL DATA



Supplemental Figure 1: Presence of ghrelin system components in normal GEP, adjacent non-tumor tissue and GEP-NETs. The graphs indicate the percentage of samples (normal GEP control, adjacent non-tumor tissue and tumoral tissue) positive for the expression of each of the ghrelin system components.



Supplemental Figure 2: Expression of ghrelin system components in GEP-NETs of different grade. The absolute mRNA expression of the different components of the ghrelin system was analyzed in grade 1, 2 and 3 GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean ± SEM.



Supplemental Figure 3: Expression of ghrelin system components in pancreas and gastrointestinal NETs. The absolute mRNA expression of the different components of the ghrelin system was analyzed in pancreas and gastrointestinal NETs samples. Data represent the mean±SEM. Asterisks (**,p<0.01; ***, p<0.001) indicate significant changes by non-paired analysis.

PART II

Novel therapeutic options for neuroendocrine tumors

Chapter 5

Effects of ketoconazole on ACTH-producing and non-ACTH-producing neuroendocrine tumor cells

Aura D Herrera-Martínez[1,2,3], Richard A. Feelders[1], Wouter W. de Herder[1], Justo P. Castaño[2], María Ángeles Gálvez Moreno[2], Fadime Dogan-Oruc[1], Rosanna van den Dungen[1] Peter M. van Koetsveld[1], Leo J. Hofland[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2]Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); Córdoba, Spain.
[3]Reina Sofia University Hospital; Córdoba, Spain.

Submitted for publication

ABSTRACT:

Prolonged remission of hypercortisolism with steroidogenesis inhibitors has been described in patients with ectopic adrenocorticotropic hormone (ACTH) syndrome. The antiproliferative and pro-apoptotic effect of ketoconazole in human cancer cells was previously suggested. The aim of this study was to explore the effects of ketoconazole on ACTH-producing and non-ACTH producing neuroendocrine tumor (NET) cell lines.

Materials and methods: Effects of ketoconazole alone, and in combination with somatostatin analogs, was evaluated in two human cell lines: DMS-79 (ectopic ACTH-producing small cell lung carcinoma) and BON-1 (human pancreatic NET). Total DNA measurement, apoptosis, cell cycle, chromogranin-A (CgA)/proopiomelanocortin (POMC) expression by qRT-PCR, serotonin, CgA and ACTH secretion assays were performed.

Results: In both cell lines, ketoconazole significantly suppressed cell growth in a dose and time-dependent manner. The effect in DMS-79 was primarily cytotoxic, while it was more apoptotic in BON-1 cells. Ketoconazole also induced increase in G0/G1 phase in both cell lines and arrest in phase G2/M of BON-1 cells. Ketoconazole did not affect the secretion of serotonin, CgA, ACTH or the mRNA expression of CgA and POMC. Decreased serotonin secretion was observed after the combination treatment with pasireotide.

Conclusions: These results suggest a direct effect of ketoconazole on cell proliferation, apoptosis and cell cycle in both ACTH- and non-ACTH producing NET cells.

BACKGROUND

Neuroendocrine tumors (NETs) are a heterogeneous family of neoplasms derived from neuroendocrine cells with a wide spectrum of clinical behavior [1]. NETs can occur sporadically or as a result of hereditary predisposition syndromes [2]. Their capacity to secrete peptide hormones divides them in functioning and non-functioning tumors [1]

One of the most uncommonly secreted hormones is adrenocorticotropic hormone (ACTH) [3, 4], resulting in hypercortisolism and ectopic ACTH syndrome (EAS). EAS is a form of Cushing Syndrome (CS) associated with overt malignancies or indolent tumors, including neuroendocrine tumors of the lungs, thymus, and gastrointestinal tract [3-6]. The management of patients with EAS requires strict control of hypercortisolism as soon as the diagnosis is established, in order to avoid related complications [3, 7, 8]. In EAS patients, initial resection of the primary lesion is the first-line treatment option. However, in some cases surgery is not possible or successful and in other cases the source of ectopic ACTH is not identified [8, 9]. For these patients, medical treatment, including ketoconazole, and adrenal resection represent alternative therapeutic options [10-12] .

Ketoconazole is widely used for medical treatment of CS and can improve clinical signs, symptoms and comorbidities [10]. Ketoconazole impairs adrenal and gonadal steroidogenesis by inhibiting side-chain cleavage, 17,20-lyase, and 11-β hydroxylase enzymes [13]. While ketoconazole seems less effective in controlling hypercortisolism in EAS patients compared to Cushing disease patients [10], the drug could exert additive effects in the control of patients with severe hypercortisolemia [13]. A direct effect ketoconazole on tumoral ACTH secretion has been suggested [14, 15], since its use in some EAS cases has been followed by prolonged remission of hypercortisolemia [14-16]. Ketoconazole also reduces ACTH secretion from thymic tumor cells in culture [17], suggesting a dual (direct/indirect) action of ketoconazole on EAS tumors.

Some ectopic ACTH-producing tumors express somatostatin receptors, suggesting a putative role of somatostatin analogs (SSA) for reducing ACTH production. Octreotide inhibits the release of ACTH in some patients with Nelson's syndrome and EAS from metastatic gastroenteropancreatic-NETs, small cell lung carcinoma, carcinoids and medullary thyroid carcinoma [18-20]. However, in the clinical practice the effect of SSA is limited, but their use has been suggested when other inhibitors of steroidogenesis fail or when parenteral administration is required [21].

Despite the development new therapeutic options in NETs, treatment strategies are still limited and unsatisfactory. As such, it is still necessary to develop novel therapeutic strategies. In this scenario, we aimed to evaluate the effect of ketoconazole as monotherapy, as well as in combination with SSA on proliferation and hormonal secretion of ACTH-producing and non-ACTH-producing neuroendocrine tumor cell lines, in order to determine potential additional direct effects of ketoconazole on NET cells.

MATERIALS AND METHODS

Cell culture

We used the human small cell lung carcinoma cell line DMS-79 and the human pancreatic neuroendocrine tumor cell line BON-1. The cell line DMS-79 (ATCC-CRL-2049™, Manassas, VA, USA) was isolated from pleural fluid of an ACTH producing small cell lung carcinoma. The BON-1 cell line was a kind gift of Dr. Townsend (The University of Texas Medical Branch, Galveston, Tex., USA) and was established from a lymph node metastasis of a human functional pancreatic neuroendocrine tumor. Both cells lines were routinely cultured in 75cm^2 flasks (Greiner bio-one, The Netherlands) at 37°C in a 5% CO_2 incubator.
BON-1 cells were cultured in DMEM/F12 (GIBCO Biocult Europe, Breda, The Netherlands) containing 10% FCS, L-glutamine, fungizone (0.5 mg/L) and penicillin (100U/mL) (Bristol-Myers Squibb, Woerden, The Netherlands). Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 85%. For the serotonin and chromogranin measurements, BON-1 cells were cultured in DMEM/F12 (GIBCO Biocult Europe, Breda, The Netherlands) containing 0.1% BSA, L-glutamine, fungizone (0.5 mg/L) and penicillin (10^5U/L) (Bristol-Myers Squibb, Woerden, The Netherlands) after an initial incubation period of 24 hours in 10% FCS to allow the cells to attach.
DMS-79 cells were cultured in RPMI 1640 (GIBCO Biocult Europe, Breda, The Netherlands) containing 10% heat inactivated (Hi)-FCS, L-glutamine, and penicillin (10^5U/L) (Bristol-Myers Squibb, Woerden, The Netherlands). Cells were harvested with pipetting and cell viability always exceeded 80%. All cell line proliferation experiments were performed at least four times.

Drugs and reagents

Ketoconazole was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and dissolved in ethanol as a 10^{-2} M stock solution (stored at -20 °C) and diluted in ethanol prior to use. We tested concentrations ranging between 10^{-5} and 10^{-7} M in BON-1 cells and concentrations ranging between $5x10^{-5}$ and $5x10^{-7}$M in DMS-79 cells, control wells were treated with vehicle only (ethanol). Octreotide and pasireotide were obtained from Novartis Pharma (Basel, Switzerland) and diluted in medium until a final concentration of 10^{-8} M.

Cell proliferation assay

Measurement of total DNA content (measure of total cell number):
Cells were plated in 1 ml medium in 24-well plates at a density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment (50.000 cells/well for three days and 10.000 cells/well for seven days in BON-1 cells; 150.000 cells/well for three days

and 75.000 cells/well for seven days in DMS-79 cells). 24 hours later, medium was refreshed and increasing concentrations of ketoconazole, alone or combined with SSA, were added to the cells.

After three and seven days of treatment, the cells were harvested for DNA measurement, as a measure of cell number. For seven days treatment medium and drugs were refreshed at day 3. The procedure for the DNA measurement has been previously described in detail [22, 23]. Briefly, the cells were treated with 150 µL of ammonia solution (1 mol/l) - Triton X 100 (0.2% v/v) for 15 minutes. Thereafter, sonification was performed (Soniprep 150; amplitude 1400 microns). Subsequently, 1 ml of assay buffer (100 mM NaCl, 100 mM EDTA, 10 mM Tris; pH 7.0) was added. For DNA measurement, 20 µl of the solution was mixed with 200 µL of Hoechst dye H33258 solution (1 µg/ml). Fluorescence was measured with the excitation and emission wavelengths set at 350 and 455 nM respectively. The fluorescence of experimental samples was referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma-Aldrich (Zwijndrecht, The Netherlands).

Quantitative RT-PCR

mRNA expression of chromoganin A (CgA) in BON-1 cells, proopiomelanocortin (POMC) in DMS-79 cells, and somatostatin receptors (SST_1, SST_2, SST_3, SST_5) in both cell lines was evaluated by quantitative RT-PCR. We used a previously described method [24]. In short, poly(A^+) mRNA was isolated using Dynabeads Oligo (Deoxythymidine)$_{25}$ (Dynal AS, Oslo, Norway). The poly(A^+) mRNA was eluted in H_2O (65 ºC) twice for 2 min each and used for cDNA synthesis in a Tris buffer [50 mm Tris-HCl (pH 8.3), 100 mm KCl, 4 mm dithiothreitol, and 10 mm $MgCl_2$] with 10 U ribonuclease inhibitor, 2 U avian myeloblastosis virus Super Reverse Transcriptase, and 1 mm of each deoxynucleotide triphosphate in a final volume of 40 µl. This mixture was incubated for one hour at 42ºC, and the resulting cDNA was diluted 5-fold in 160 µl sterile H_20. The total reaction volume (25 µl) consisted of 10 µl cDNA and 15 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ). Primers were used at final concentration of 300 nm and probe at 200 nm. Real-time qPCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche) and the ABI Prism 7700 Sequence Detection System (PerkinElmer, Foster City, CA). After two initial heating steps at 50ºC (2 min) and 95ºC (10 min), samples were subjected to 40 cycles of denaturation at 95ºC (15 sec) and annealing at 60ºC (60 sec). All samples were assayed in duplicate. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set [25]. To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template.

CgA and POMC mRNA were normalized to the housekeeping genes *HPRT1*, beta-glucuronidase (*GUSB*) and beta-actine (*ACTB/ACTB*) expression levels using the method

of Vandesompele [26]; somatostatin receptors were normalized against *HPRT1*. *CgA, POMC, GUSB, ACTB* primer/probes were purchased from Thermo Fisher Scientific, The Netherlands, while the used sequence of somatostatin receptors and *HPRT1* has been previously described [27]. PCR efficiencies were as follows: *CgA*: 1.95, *POMC*: 1.92, $SSTR_1$: 2, $SSTR_2$: 1.91, $SSTR_3$: 1.92, $SSTR_5$: 1.92, *HPRT1*: 1.91, *ACTB*: 1.91, *GUSB*: 2. The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta C1}$[28], after efficiency correction [29] of target and reference gene transcripts (*HPRT, GUS-B, ACTB*). All experiments were performed at least twice with four replicates.

Cell cycle assay – apoptosis analysis

Cells were plated in 12-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment. 24 hours later, ketoconazole was added to wells in triplicate. In each cell line, the effects of ketoconazole on cell cycle were tested in three concentrations ($5x10^{-6}$, 10^{-6} and $5x10^{-5}$ M in DMS-79; 10^{-6}, $5x10^{-6}$ and 10^{-5} M in BON-1 cells) on cell proliferation after 7 days of treatment. We evaluated the effects of the compounds on cell cycle and apoptosis after 72 h of treatment. Following treatment, cells were harvested and collected by centrifugation. For cell cycle, cells were fixed in 70% ice-cold ethanol, followed by an overnight incubation at -20ºC. Samples were measured using the Muse™ cell cycle kit (Merck) and prepared according the manufacturers protocol. For apoptosis measurement, cells were re-suspended in 100 µl PBS/1% FCS, according to the protocol provided by the manufacturer. A commercially available kit was used (Muse™ annexin V dead cell). All measurements were performed using the Muse® Cell Analyzer (Merck). Apoptosis was measured using a commercially available Elisa (Cell Death Detection ELISA Plus, Roche, The Netherlands) according to the manufacturer's protocol. Lactate dehydrogenase LDH release was measured using a kit (pierce LDH cytotoxicity assay kit, Thermo scientific, The Netherlands) and samples were treated according to the protocol provided by the manufacturer. All experiments were performed at least three times using three replicates.

ACTH secretion assay

In DMS-79 cells, we evaluated the effects of ketoconazole alone and in combination with octreotide/pasireotide on ACTH secretion after 3 and 7 days of treatment. Cells were plated in 24-well plates and treated as described earlier for the cell proliferation assay. The experiment was performed twice. ACTH levels were determined using a chemiluminescent immunometric assay in an IMMULITE® 2000 system. ACTH secretion values were normalized for cell amount per well (DNA). The sensitivity of this assay ranged between 1.1 -270 pmol/L. Untreated control values were within this range. All experiments were performed at least two times using four replicates.

Chromogranin A and serotonin secretion assay

BON-1 cells were plated in 24-well plates using medium containing 0.1% BSA, and treated as described earlier for the cell proliferation assay. Medium was collected from each well, to samples for the serotonin Elisa ascorbic acid was added (final concentration 0.1%). A commercially available human chromogranin A ELISA kit (Epitope diagnostics Inc, San Diego CA, USA) and a serotonin high sensitive ELISA (IBL international, Hamburg Germany), were used following the manufacturer's instructions. The DNA content per well was used to correct both serotonin and CgA absolute values. All experiments were performed at least two times using four replicates.

Statistical analysis

For the statistical analysis, statistical software of GraphPad Prism version 5 (GraphPhad Software, San Diego, CA) was used. Between-group comparisons were analyzed by the Mann–Whitney U test (nonparametric data), or the Kruskal–Wallis test (nonparametric data, when we compared more than two groups). Differences were taken to be statistically significant at $p<0.05$. Results are expressed as mean ± S.E.M. and percentages. Log transformation was used for calculating the IC_{50}.

RESULTS

Effects of ketoconazole

The growth inhibitory effects of ketoconazole on DMS-79 and BON-1 cells growth are dose- and time-dependent (Figure 1).

DMS-79 cells (Figure 1A) are slightly less sensitive to ketoconazole compared with BON-1 cells (Figure 1B). The effects of ketoconazole ranged between 47.20% inhibition ($p<0.0001$) at the maximal dose ($5x10^{-5}$ M) and 10.32% ($p<0.05$) at the dose of $5x10^{-6}$ M after 3 days of treatment. After 7 days, the effect of ketoconazole increased to 87.3%, 46.3% and 29.8% with ketoconazole $5x10^{-5}$ M - 10^{-5} M - $5x10^{-6}$ M respectively ($p<0.0001$). The IC50 after seven days of incubation was $1.5x10^{-5}$ M (95%CI: $1.0x10^{-5}$ - $2.3x10^{-5}$ M).

In BON-1 cells, the inhibitory effect of ketoconazole ranged between 41.03% at the maximal dose (10^{-5} M; $p<0.0001$) and 19.1% at a dose of $5x10^{-6}$ M ($p<0.01$) after three days of treatment (Figure 1B). After seven days, the growth inhibitory effect increased to 95.23% and 74.82% with ketoconazole 10^{-5} M and $5x10^{-6}$ M respectively ($p<0.0001$) (Figure 1B). The IC50 after seven days of incubation was $7.7x10^{-6}$ M (95%CI: $3.8x10^{-6}$ – $1.6x10^{-5}$ M).

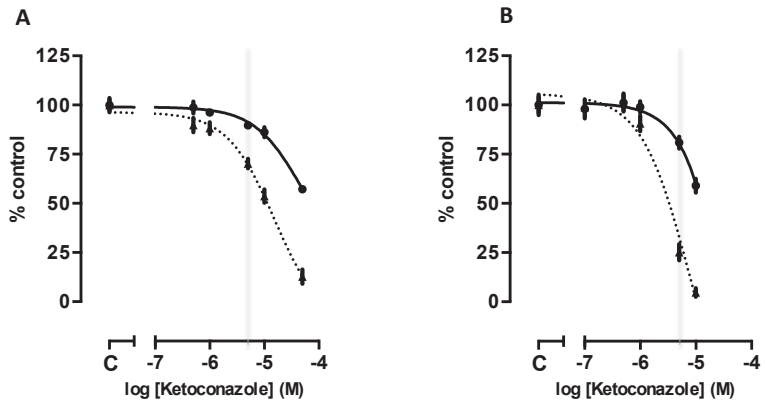


Figure 1: Dose- and time-dependent effect of ketoconazole after 3 and 7 days of treatment. (A): effect on cell growth (DNA) in DMS-79 cells (B): effect on cell growth in BON-1 cells. Ketoconazole significantly suppressed cell growth in a dose and time-dependent manner. Solid and dotted black lines represent DNA content in percentage after three (__) and seven days (…) respectively. The gray vertical dotted line represents the clinically relevant plasmatic concentration of ketoconazole. Values represent mean ± SEM and are shown as a percentage of control. C: control.

Effect of ketoconazole in combination with octreotide and pasireotide

In DMS-79 cells, neither pasireotide nor octreotide inhibited cell growth (Figure 2A and 2B, left panels). In combination with pasireotide or octreotide, ketoconazole exerted a similar growth inhibitory effect compared to the effect of ketoconazole alone (Figure 2A and 2B, right panels)

In BON-1 cells, octreotide slightly decreased cell growth by 9.4% ($P<0.05$) after 3 days of treatment only (Figure 2C, left panel), whereas pasireotide slightly inhibited cell growth by 13.5% ($p<0.01$) at day 7 (Figure 2D, left panel). In combination with pasireotide and octreotide, ketoconazole appeared slightly less effective at high dose ketoconazole (Figure 2C and 2D, right panels).

Somatostatin receptor subtype expression

The relative mRNA expression of somatostatin receptor subtypes was evaluated in both cell lines (Supplementary Figure 1). SST_1 and SST_5 expression in DMS-79 was lower compared to BON-1 cells. Rank order of expression in DMS-79 was $SST_1 = SST_2 > SST_3 > SST_5$ and for BON-1 $SST_1 > SST_5 >> SST_2 > SST_3$.

Apoptosis assays

In DMS-79 cell line, the highest ketoconazole concentration evaluated ($5x10^{-5}$ M) induced a significant increase in dead cells ($p<0.001$) with a slight increase in late apoptosis ($p<0.05$) and decrease in living cells ($p<0.01$; Figure 3A). Moreover, the LDH/DNA ratio increased

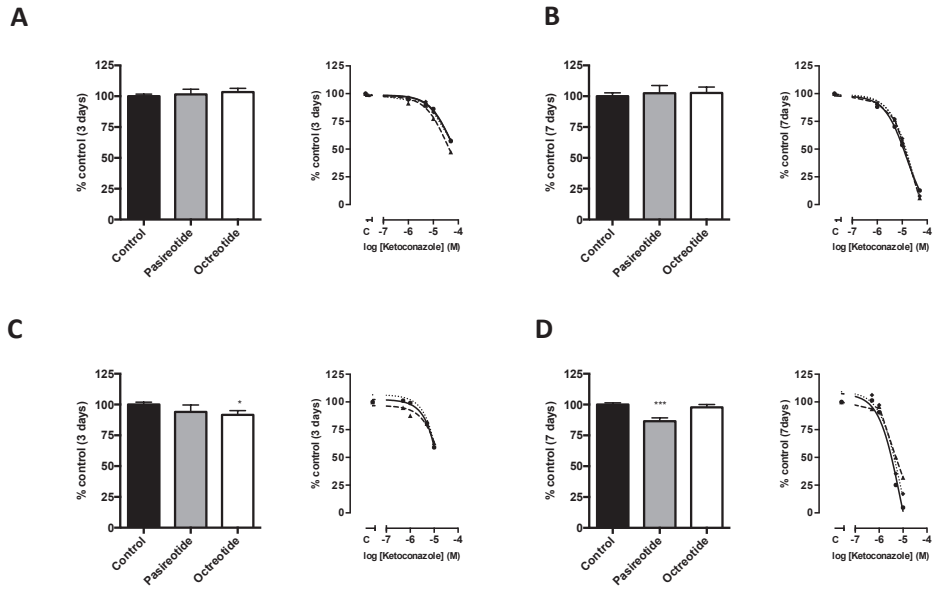


Figure 2: Dose- and time-dependent effect of the combination treatment with ketoconazole and somatostatin analogs on cell proliferation after 3 and 7 days of treatment. Panels A and B represent the percentage of control of cell growth in DMS-79 cells after three and seven days respectively; panels C and D represent the percentage of control of cell growth in BON-1 cells after three and seven days respectively. The bar graphs represent the effect of SSA alone (octreotide and pasireotide, both at 10^{-8} M), compared to untreated control. The line graphs represents ketoconazole alone, or in combination with a fixed dose (10^{-8}M) of SSA (set at 100%). Ketoconazole significantly suppressed DNA content in a dose and time-dependent manner, a slightly decreased effect was observed after combination with somatostatin analogs. Lines represent DNA content as percentage after incubation with ketoconazole alone (solid line), ketoconazole-octreotide (dashed line) and ketoconazole-pasireotide (dotted line) respectively. Values represent mean ± SEM and are shown as a percentage of control. C: control. Asterisks: *p<0.05; ***,p<0.0001 compared to untreated controls

after three days of treatment with ketoconazole $5x10^{-5}$ M and 10^{-5} M (Figure 3C left panel; p<0.001; p<0.05 respectively), while the apoptosis/DNA ratio increased only with the highest concentration tested (Figure 3C, right panel; p<0.001).

In BON-1 cells the highest ketoconazole concentration tested (10^{-5} M) induced a significant increase in early-, as well as late apoptosis (p<0.0001). Consequently, the percentage of live and dead cells decreased (Figure 3B). In order to confirm these results, the percentage of LDH release was determined. After three days of treatment with ketoconazole 10^{-5} M, LDH increased by approximately 50%, combined with a threefold increase in apoptosis/DNA ratio (p<0.05; Figure 3D).

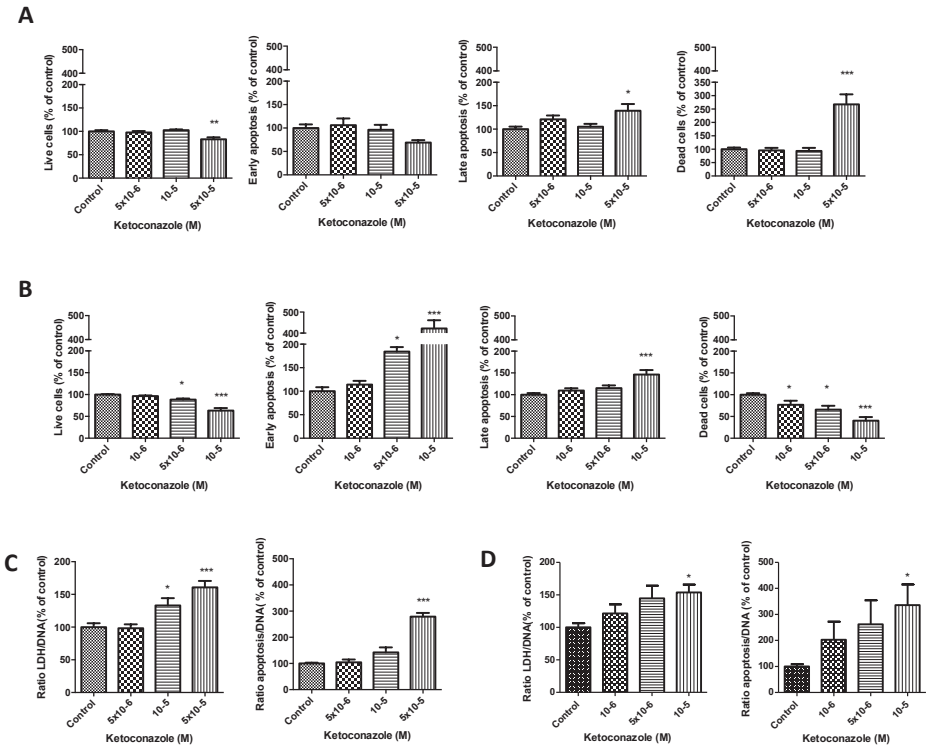


Figure 3: Dose-dependent effect of ketoconazole on apoptosis after 3 days of treatment. (A): DMS-79 cells: the highest ketoconazole concentration tested ($5x10^{-5}$ M) induced a significant increase in dead cells and late apoptosis. (B): BON-1 cells: the highest ketoconazole concentration tested (10^{-5} M) induced a significant increase in early apoptosis as well as in late apoptosis. Apoptosis (DNA fragmentation) and LDH release after 3 days of treatment in DMS-79 cells (C) and BON-1 cells (D). The highest ketoconazole concentration tested increased apoptosis as well as LDH levels in both cell lines. Values represent mean ± SEM and are expressed as percentage of control of the apoptosis/DNA ratio and LDH/DNA ratio. Asterisks: *p<0.05; **,p<0.01, ***,p<0.001 compared to untreated controls

Cell cycle progression

In DMS-79, a slight statistically significant G0/G1-phase increase was observed with the highest ketoconazole concentration tested (p<0.01), without having a statistical significant effect on the other cell cycle phases (Figure 4A).

In BON-1 cell line, ketoconazole (10^{-5} M - $5x10^{-6}$ M) induced a statistical significant G0/G1-phase increase (p<0.0001, p<0.01 respectively), accompanied by a decrease in G2-phase (p<0.0001; Figure 4B).

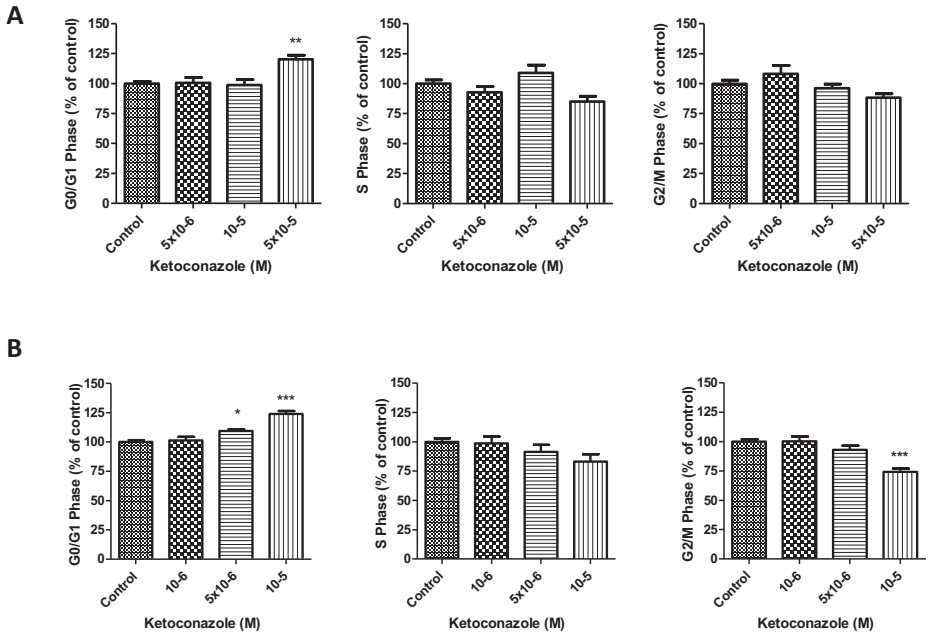


Figure 4: Dose-dependent effect of ketoconazole on cell cycle progression in DMS-79 cells (A) and BON-1 cells (B) after 3 days of treatment. In DMS-79 cells, ketoconazole ($5x10^{-5}$ M) induced a slight but significant G1-phase increase. In BON-1 cells, ketoconazole (10^{-5} – $5x10^{-6}$ M) induced G1-phase increase and decrease in G2-phase. Asterisks: *p<0.05; **,p<0.01, ***,p<0.001 compared to untreated controls

Chromogranin A and POMC expression

Ketoconazole did not have a statistically significant effect on POMC mRNA expression in DMS cells (Supplemental Figure 2A), nor on CgA mRNA expression in BON-1 cells (Supplemental Figure 2B) after three days of treatment at any of the doses tested.

ACTH and serotonin secretion

Effects on ACTH secretion were evaluated in DMS-79 cells. When corrected for the effect of ketoconazole on total cell number (ACTH/DNA ratio), ketoconazole inhibited the release of ACTH after three days of treatment with the highest tested concentration of ketoconazole (5x10-5 M) (Figure 5A). ACTH was undetectable after seven days of treatment with ketoconazole $5x10^{-5}$ M (Figure 5B), likely due to the very potent lowering of cell number. When combined with pasireotide, a similar pattern of inhibition of ACTH release was observed (Figure 5A and 5B, middle panels). Octreotide alone, slightly inhibited ACTH release after 7 days of incubation. In combination with ketoconazole, only in the presence of the highest concentration of ketoconazole ACTH concentrations were undetectable (figure 5A and 5B, right panels). Supplementary figure 3 shows the effect of ketoconazole

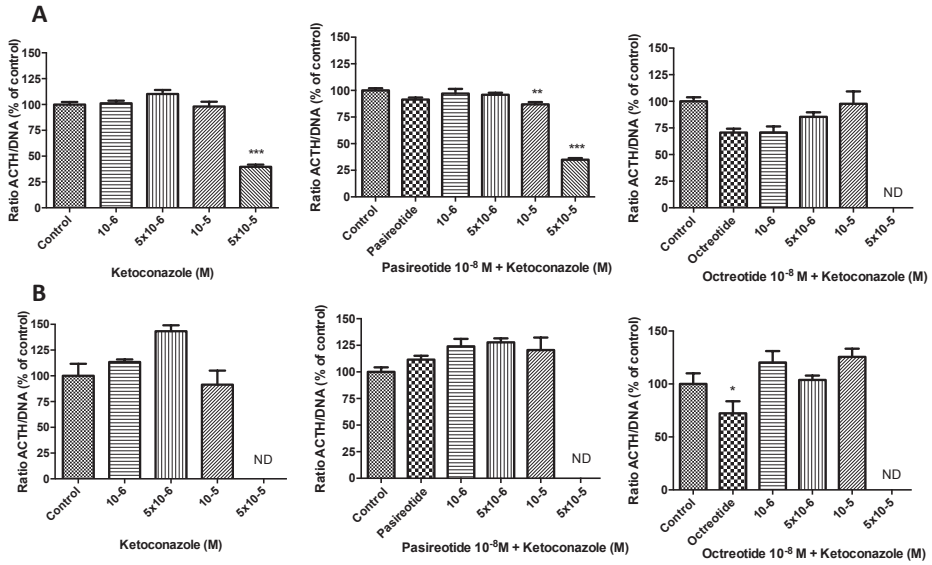


Figure 5: Dose-dependent effect of ketoconazole on ACTH secretion in DMS-79 cells after 3 (A) and 7 days (B) of treatment. Ketoconazole ($5x10^{-5}$ M) significantly decreased ACTH secretion (corrected for cell number) after three days but not after seven days of treatment, as well as its combination with pasireotide; octreotide decreased the ACTH/DNA ratio after seven days. ACTH secretion was normalized to the effect on cell number (ratio ACTH/DNA). ND: non-detectable ACTH. Asterisks: *p<0.05; **,p<0.01; ***,p<0.001, compared to untreated controls.

on ACTH concentrations in the medium, not corrected for the effect of ketoconazole on cell number.It was not possible to measure serotonin secretion in BON-1 cells cultured in medium with FCS, due to a matrix effect in the serotonin assay (data not shown). In order to determine the effect of ketoconazole on serotonin secretion, BON-1 cells were cultured in medium containing 0.1% BSA. A dose-dependent inhibitory effect of ketoconazole on the proliferation of BON-1 cell line in 0.1% BSA medium was observed (Supp. figure 4A). The absolute production of serotonin decreased when cells were treated with high doses of ketoconazole, especially when combined with pasireotide, while no additional effect was observed when combined with octreotide (Supp. figure 4B). After calculating the serotonin/DNA ratio (to correct for the effect of ketoconazole on cell number), ketoconazole, and its combination with octreotide, did not have a statistical significant effect on serotonin secretion, while pasireotide decreased its production by 45%. (Figure 6A).

The absolute production of CgA in BON-1 cells decreased when cells were treated with high doses of ketoconazole (10^{-5}-$5x10^{-6}$ M). This effect remained when combined with pasireotide and octreotide (Supp. figure 4C). After calculating the CgA/DNA ratio, no statistical significant effect was observed after three days of treatment with ketoconazole alone or in combination with SSA (Figure 6B).

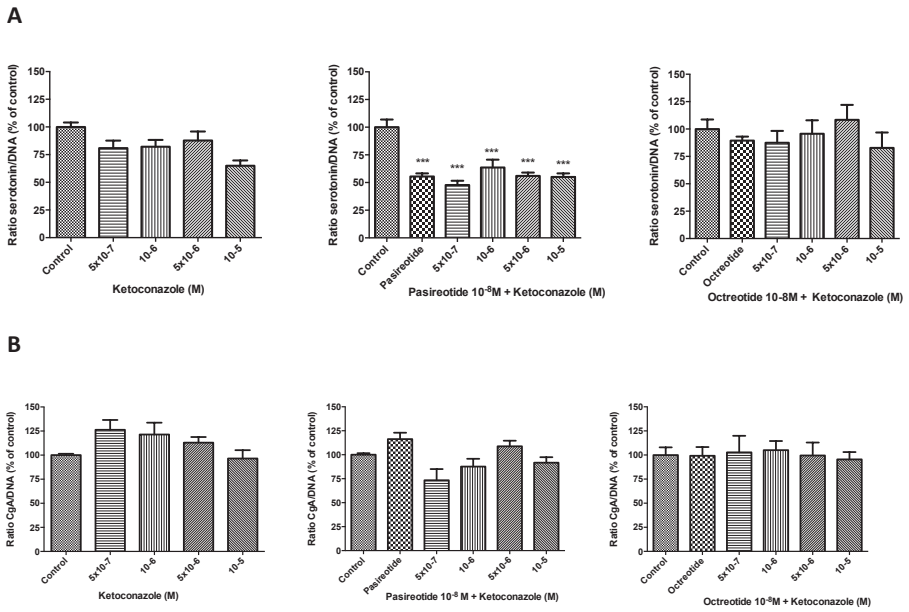


Figure 6: Dose-dependent effect of ketoconazole on serotonin (A) and chromogranin A (B) secretion in BON-1 cells after 3 days of treatment. In BON-1 cells, pasireotide reduced serotonin secretion; ketoconazole alone or in combination with octreotide did not significantly affected serotonin secretion. No statistically significant effect was observed on CgA secretion after treating cells with ketoconazole alone or in combination with pasireotide or octreotide. Serotonin and CgA secretion were normalized to the effect on cell number (ratio serotonin/DNA; ratio CgA/DNA). Asterisks: ***,p<0.001, compared to untreated controls.

DISCUSSION

In the present study, we evaluated the direct effects of ketoconazole in ACTH-producing and non-ACTH-producing NET cell lines. In this respect, we examined effects on proliferation, mRNA expression and secretion by ketoconazole as monotherapy, as well as in combination with SSA, the latter representing the recommended first line therapy in non-functioning/functioning progressive G1/G2 NETs [30]. Our results revealed a particular effect of ketoconazole on proliferation, cell cycle, apoptosis and mRNA expression, which was slightly different between the evaluated NET cell lines.

In our study, we included clinically relevant concentrations of ketoconazole, based on previous results in humans. Several studies report serum ketoconazole levels of 3.6 µg/mL and 6.5 µg/mL after the administration of 200 and 400 mg of ketoconazole respectively [31]. The continuous administration of 400 mg/8 hours resulted in serum ketoconazole levels of 2-9 µg/mL [32]. Several effects in this study on the NET cell lines have been achieved with these ketoconazole concentrations (5.3 µg/mL ketoconazole corresponds to 10^{-5} M).

Sharma and Nieman described four patients with EAS with prolonged remission of hypercortisolism following treatment with ketoconazole alone or in combination with metyrapone and/or mitotane. In one patient this was accompanied by increased ACTH levels indicating a direct, sustained toxic effect on the adrenal cortex. In two other patients, however, ACTH levels were normal to low suggesting a direct effect of ketoconazole on ectopic ACTH producing tumor cells [16]. In this sense, we observed decreased proliferation rate in both ACTH-producing and non-ACTH-producing NET cell lines when exposed to ketoconazole, including clinically relevant concentrations ($5x10^{-6}$ M). Important to note, DMS-79 cells are slightly less sensitive than BON-1 cells, and required higher doses for obtaining similar inhibitory effects.

The mechanism of action of ketoconazole seems to be different in both cell lines. In BON-1 cells, ketoconazole has an apoptotic effect (especially increasing early apoptosis), whereas in DMS-79 cell line the effect seems to be more cytotoxic.

Previous reports have described a cytotoxic effect of ketoconazole in prostate cancer, adrenal cancer, and male metastatic breast cancer cell lines with a dose- and time-dependent pattern at clinically feasible concentrations [33]. In addition, it has been shown that ketoconazole decreased tumor surface area and tumor colonies in liver metastasis of human pancreatic adenocarcinoma in animal models [34]. Due to its androgen lowering properties, ketoconazole is also evaluated as a potential treatment in other neoplasms such as prostate cancer [32]. In these patients, the drug is administered at high doses (400 mg/8 hours/day), in combination with hydrocortisone [35]. Approximately 27-60% of treated patients show up to 50% decrease in prostate antigen without receiving concomitant anti-androgen treatment. In addition, those patients that respond to treatment and do not have metastatic disease, showed prolonged responses even after seven years [35, 36]. Related to this, preliminary reports have suggested that the use of ketoconazole (600 mg/day) combined with docetaxel has a significant antitumor effect in castration resistant prostate cancer patients [37]. The cell growth inhibitory effects observed in our study suggest that a direct antitumor action of ketoconazole may be involved in the sustained remission which is sometimes observed in patients with ectopic ACTH secreting NET that are treated with this drug (13).

Focusing on treatment for NETs, it is already known that SSA have a weak antitumor efficacy, but they are useful for symptoms control and disease stabilization [30]. In our study we observed a very weak anti-proliferative effect of octreotide after three days and of pasireotide after seven days of treatment, which is concordant with literature reports [30]. The response to pasireotide after seven days in BON-1 cells is probably related to the high expression of SST_1 and SST_5 receptors in this cell line. Interestingly, in BON-1 cells, the effectiveness of ketoconazole in combination with SSA on cell proliferation seems to be slightly decreased.

Our results on apoptosis are concordant with previous studies in other cell line models. Ho et al. reported apoptosis induced by ketoconazole in human colorectal and hepatocellular carcinoma cell lines through the p53 pathway (increased *bax* and decreased *bcl-2* gene products) with minimal ketoconazole concentrations of 5 µg/mL [38]. Similarly, Won et al. suggested

that ketoconazole could increase apoptosis and decrease cell viability in rats through reactive oxygen species (ROS) in rat cardiomyocytes [39]. Some other signaling routes have been proposed for explaining the apoptotic effect of ketoconazole, including the JNK phosphorylation in human osteosarcoma [40]. Ketoconazole, when combined with terfenadine, seems to potentiate the inhibition of cytochrome p450 3A4 in human cancer cell lines [41].

When evaluating cell cycle, published results are contradictory. Forgue-Lafitte et al. reported decreased cell number in S phase and a corresponding increase in G0-G1 phases in colon cancer cells treated with ketoconazole [42]. In contrast, Chen et al. described G0/G1 arrest in human colorectal and hepatocellular cell lines [43]. However, in our study, we observed in both cell lines a significant increase in G0/G1 phase, which was accompanied with an arrest of G2/M phases in BON-1 cells. These results suggest that ketoconazole may have a differential mechanism of action in different tumor cell lines.

When analyzing secretion, ketoconazole did not affect serotonin secretion, but the effect of pasireotide on serotonin production is relevant and stable after its combination with ketoconazole, suggesting a therapeutic indication for pasireotide in patients with carcinoid syndrome or functioning NETs with liver metastasis. Interestingly, pasireotide had no effect on CgA secretion, suggesting that serotonin and not CgA is a marker of secretion. Pasireotide has a higher binding affinity for SST_1, SST_3 and SST_5 when compared with octreotide, as well as a slightly lower affinity for SST_2 [44]. Based on this, pasireotide has the capacity of suppressing pituitary hormone release for prolonged periods of time [44, 45] due to central and peripheral (liver) regulation [44], which could be a potential explanation for its effect on serotonin secretion. Moreover, as we did not observe any effect of ketoconazole alone or combined with SSA on CgA secretion after correcting for its effect on cell number, it is likely that CgA is related to tumor growth and not to tumor secretion capacity [46].

Changes on hormone secretion by ketoconazole have been previously described. Ketoconazole increases the spontaneous and stimulated release of prolactin in normal and tumoral rat pituitary cells [47], and decreases the ACTH production in human thymic carcinoid cells [17]. It has been also hypothesized that ketoconazole could affect tumoral ACTH and cyclic ACTH secretion in vivo [16]. In our study, however, we did not observe a direct inhibitory effect of clinically feasible concentrations of ketoconazole on ACTH production by DMS-79 cells (corrected for the effect of ketoconazole on cell growth), suggesting that the effect of ketoconazole is primarily cytotoxic.

In summary, this study provides a primary comprehensive mapping of the effect of ketoconazole on NET cell lines, suggesting a potential effect of ketoconazole on cell proliferation, apoptosis and cell cycle. This antitumor effect appears not tumor cell type specific, since it was observed in both ACTH secreting and non-ACTH secreting NET cells. A direct antitumor action of ketoconazole may be an explanation for prolonged remission of hypercortisolemia observed in some patients with EAS and may have an added therapeutic value next to its adrenal-suppressive effects. Additional studies, including primary (ACTH-producing) NET cultures, are required to confirm and further extend these results.

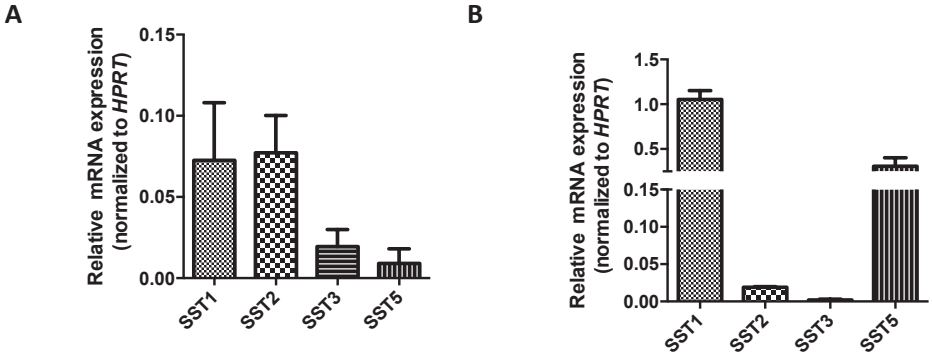
REFERENCES:

1. Hofland, J., et al., *Recent developments in the diagnosis and therapy of well-differentiated neuroendocrine tumours.* Neth J Med, 2018. **76**(3): p. 100-108.
2. Fraenkel, M., et al., *Incidence of gastroenteropancreatic neuroendocrine tumours: a systematic review of the literature.* Endocr Relat Cancer, 2014. **21**(3): p. R153-63.
3. Herrera-Martinez Aura D, P.C.J.C., Bahamondes Opazo Rodrigo, Barrera Martín Ana, Rebollo Roman Angel, Díaz Iglesia Carlos, Gálvez Moreno María A, *ACTH Producing Pancreatic Neuroendocrine Tumors in Multiple Endocrine Neoplasia Type 1.* Journal of the pancreas, 2017. **02**: p. 175-180.
4. Kaltsas, G., et al., *Paraneoplastic syndromes secondary to neuroendocrine tumours.* Endocr Relat Cancer, 2010. **17**(3): p. R173-93.
5. Fazel, P., et al., *The ectopic adrenocorticotropic hormone syndrome in carcinoid tumors.* Proc (Bayl Univ Med Cent), 2008. **21**(2): p. 140-3.
6. Kamp, K., et al., *Prevalence and clinical features of the ectopic ACTH syndrome in patients with gastroenteropancreatic and thoracic neuroendocrine tumors.* Eur J Endocrinol, 2016. **174**(3): p. 271-80.
7. Kondo, T., et al., *A case of ectopic adrenocorticotropic hormone-producing pancreatic neuroendocrine tumor with multiple liver metastases.* Endocr J, 2010. **57**(3): p. 229-36.
8. Lacroix, A., et al., *Cushing's syndrome.* Lancet, 2015. **386**(9996): p. 913-27.
9. Wajchenberg, B.L., et al., *Ectopic adrenocorticotropic hormone syndrome.* Endocr Rev, 1994. **15**(6): p. 752-87.
10. Nieman, L.K., et al., *Treatment of Cushing's Syndrome: An Endocrine Society Clinical Practice Guideline.* J Clin Endocrinol Metab, 2015. **100**(8): p. 2807-31.
11. Isidori, A.M., et al., *The ectopic adrenocorticotropin syndrome: clinical features, diagnosis, management, and long-term follow-up.* J Clin Endocrinol Metab, 2006. **91**(2): p. 371-7.
12. Alberda, W.J., et al., *Endoscopic bilateral adrenalectomy in patients with ectopic Cushing's syndrome.* Surg Endosc, 2012. **26**(4): p. 1140-5.
13. Feelders, R.A. and L.J. Hofland, *Medical treatment of Cushing's disease.* J Clin Endocrinol Metab, 2013. **98**(2): p. 425-38.
14. Beardwell, C.G., A.R. Adamson, and S.M. Shalet, *Prolonged remission in florid Cushing's syndrome following metyrapone treatment.* Clin Endocrinol (Oxf), 1981. **14**(5): p. 485-92.
15. Loh, K.C., R. Gupta, and A.H. Shlossberg, *Spontaneous remission of ectopic Cushing's syndrome due to pheochromocytoma: a case report.* Eur J Endocrinol, 1996. **135**(4): p. 440-3.
16. Sharma, S.T. and L.K. Nieman, *Prolonged remission after long-term treatment with steroidogenesis inhibitors in Cushing's syndrome caused by ectopic ACTH secretion.* Eur J Endocrinol, 2012. **166**(3): p. 531-6.
17. Steen, R.E., et al., *In vivo and in vitro inhibition by ketoconazole of ACTH secretion from a human thymic carcinoid tumour.* Acta Endocrinol (Copenh), 1991. **125**(3): p. 331-4.
18. Lamberts, S.W., et al., *A role of (labeled) somatostatin analogs in the differential diagnosis and treatment of Cushing's syndrome.* J Clin Endocrinol Metab, 1994. **78**(1): p. 17-9.
19. de Herder, W.W., A.J. van der Lely, and S.W. Lamberts, *Somatostatin analogue treatment of neuroendocrine tumours.* Postgrad Med J, 1996. **72**(849): p. 403-8.
20. de Herder, W.W., et al., *Somatostatin receptor scintigraphy: its value in tumor localization in patients with Cushing's syndrome caused by ectopic corticotropin or corticotropin-releasing hormone secretion.* Am J Med, 1994. **96**(4): p. 305-12.

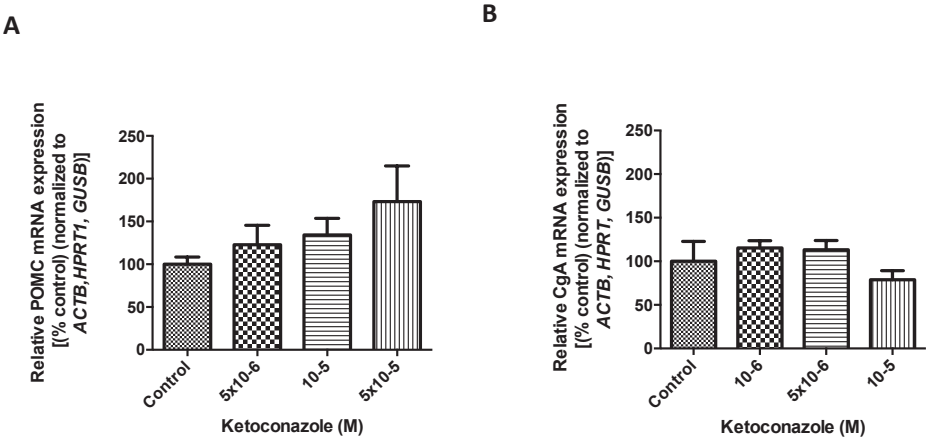
21. Uwaifo, G.I., et al., *Is there a therapeutic role for octreotide in patients with ectopic Cushing's syndrome?* J Endocrinol Invest, 2003. **26**(8): p. 710-7.
22. Hofland, L.J., P.M. van Koetsveld, and S.W. Lamberts, *Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995.* Eur J Cancer, 1990. **26**(1): p. 37-44.
23. Downs, T.R. and W.W. Wilfinger, *Fluorometric quantification of DNA in cells and tissue.* Anal Biochem, 1983. **131**(2): p. 538-47.
24. Ferone, D., et al., *Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes.* Am J Physiol Endocrinol Metab, 2002. **283**(5): p. E1056-66.
25. R, R., *Quantification on the LightCycler.* In: Meuer S, Wittwer C and Nakagawara K, eds. Rapid cycle real-time PCR, methods and applications. Heidelberg: Springer Press, 2001: p. 21-34.
26. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. RESEARCH0034.
27. de Bruin, C., et al., *Coexpression of dopamine and somatostatin receptor subtypes in corticotroph adenomas.* J Clin Endocrinol Metab, 2009. **94**(4): p. 1118-24.
28. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.
29. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
30. Oberg, K., et al., *Neuroendocrine gastro-entero-pancreatic tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2012. **23 Suppl 7**: p. vii124-30.
31. Van Tyle, J.H., *Ketoconazole: Mechanism of action, spectrum of activity, pharmacokinetics, drug interactions, adverse reactions and therapeutic use.* Pharmacotherapy, 1984. **4**: p. 343–347.
32. Heyns, W., et al., *Endocrine effects of high-dose ketoconazole therapy in advanced prostatic cancer.* Acta Endocrinol (Copenh), 1985. **110**(2): p. 276-83.
33. Rochlitz, C.F., et al., *Cytotoxicity of ketoconazole in malignant cell lines.* Cancer Chemother Pharmacol, 1988. **21**(4): p. 319-22.
34. Tzanakakis, G.N., K.C. Agarwal, and M.P. Vezeridis, *Inhibition of hepatic metastasis from a human pancreatic adenocarcinoma (RWP-2) in the nude mouse by prostacyclin, forskolin, and ketoconazole.* Cancer, 1990. **65**(3): p. 446-51.
35. Huguet Pérez J, M.R.P., Palou Redorta J, Villavicencio Mavrich H, *Cáncer de próstata hormonorresistente. Cambios en las estrategias terapéuticas desde la demostración de la utilidad de la quimioterapia.* Actas Urol Esp, 2006. **30 (2)**: p. 123-133.
36. Scholtz M, J.R., Strum S, Brosman S, Johnson H, Lam R., *Long-term outcome with androgen independent prostete cancer treated with ketoconazole and hidrocortisone.* Journal of Urology, 2005. **173(6)**: p. 1947-1952.
37. Figg, W.D., et al., *A phase I clinical study of high dose ketoconazole plus weekly docetaxel for metastatic castration resistant prostate cancer.* J Urol, 2010. **183**(6): p. 2219-26.
38. Ho, Y.S., et al., *Ketoconazole-induced apoptosis through P53-dependent pathway in human colorectal and hepatocellular carcinoma cell lines.* Toxicol Appl Pharmacol, 1998. **153**(1): p. 39-47.
39. Won, K.J., et al., *Ketoconazole induces apoptosis in rat cardiomyocytes through reactive oxygen species-mediated parkin overexpression.* Arch Toxicol, 2015. **89**(10): p. 1871-80.
40. Lin, K.L., et al., *Ketoconazole-induced JNK phosphorylation and subsequent cell death via apoptosis in human osteosarcoma cells.* Toxicol In Vitro, 2009. **23**(7): p. 1268-76.

41. Wang, Y.J., et al., *Ketoconazole potentiates terfenadine-induced apoptosis in human Hep G2 cells through inhibition of cytochrome p450 3A4 activity.* J Cell Biochem, 2002. **87**(2): p. 147-59.
42. Forgue-Lafitte, M.E., et al., *Effects of ketoconazole on the proliferation and cell cycle of human cancer cell lines.* Cancer Res, 1992. **52**(24): p. 6827-31.
43. Chen, R.J., et al., *Ketoconazole induces G0/G1 arrest in human colorectal and hepatocellular carcinoma cell lines.* Toxicol Appl Pharmacol, 2000. **169**(2): p. 132-41.
44. van der Hoek, J., et al., *The somatostatin analogue SOM230, compared with octreotide, induces differential effects in several metabolic pathways in acromegalic patients.* Clin Endocrinol (Oxf), 2005. **63**(2): p. 176-84.
45. Bruns, C., et al., *SOM230: a novel somatostatin peptidomimetic with broad somatotropin release inhibiting factor (SRIF) receptor binding and a unique antisecretory profile.* Eur J Endocrinol, 2002. **146**(5): p. 707-16.
46. Colombo, B., et al., *Chromogranin A expression in neoplastic cells affects tumor growth and morphogenesis in mouse models.* Cancer Res, 2002. **62**(3): p. 941-6.
47. Drouhault, R., et al., *Differential effects of ketoconazole on prolactin and growth hormone release by normal and tumoral rat anterior pituitary cells in vitro.* Neuroendocrinology, 1989. **50**(5): p. 513-8.

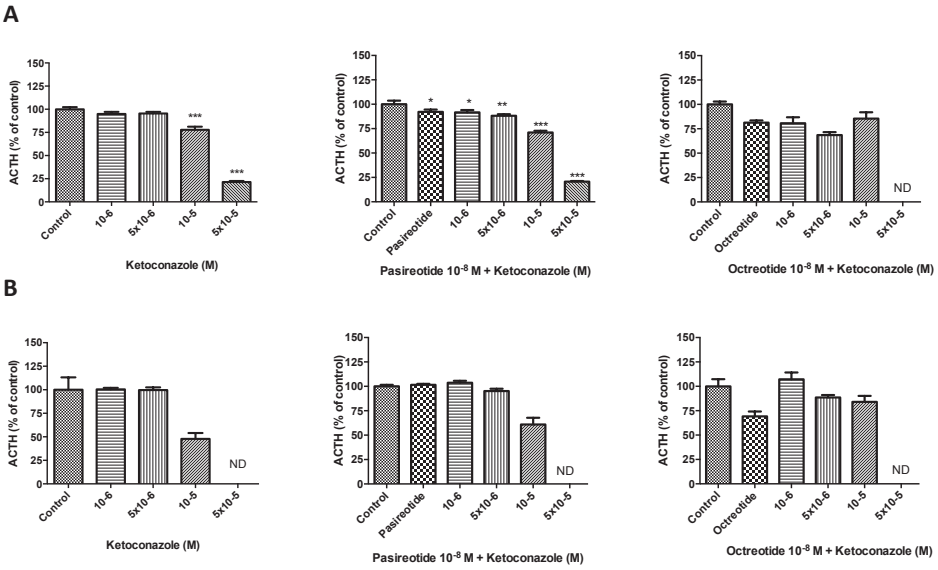
SUPPLEMENTAL DATA



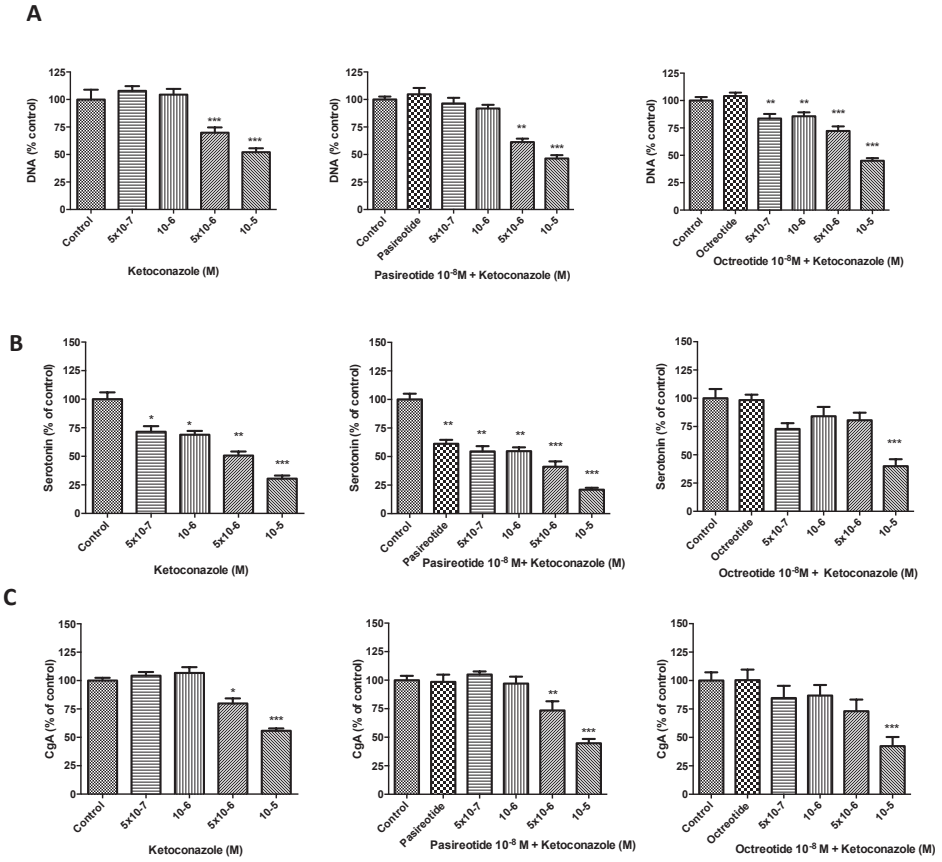
Supplemental Figure 1: mRNA expression profile of somatostatin receptors in DMS-79 cells (A) and BON-1 cells (B). BON-1 cells express predominantly SST_5 followed by SST_1. SSTs expression in DMS-79 is lower than in BON-1 cells, SST_2 is the most expressed receptor by SST_1 and SST_3.



Supplemental Figure 2: Effect of ketoconazole on relative POMC expression in DMS-79 cells (A) and CgA mRNA expression in BON-1 cells (B). Ketoconazole did not significantly affect POMC expression in DMS-79 or CgA expression in BON-1 cells after 3 days of treatment.



Supplemental Figure 3: Effect of ketoconzale, alone or in combination with pasireotide or octreotide (both at 10-8M) on ACTH secretion by DMS-79 cells. Values are expressed as percentage of untreated control after three **(A)** and seven **(B)** days of treatment. The absolute ACTH values of the control group after three days ranged between 10.4±2.2 – 28.2±8.4 pmol/L. After seven days, the absolute ACTH values of the control group ranged between 14.1±3.2 – 18.6±5.7 pmol/L. Asterisks: *,p<0.05; **,p<0.01; ***,p<0.001, compared to untreated controls. ND: non-detectable ACTH.



Supplemental Figure 4: (A) Dose-dependent effect of ketoconazole and combined with pasireotide and octreotide on cell proliferation in BON-1 cells, expressed as percentage of DNA content/well after three days of treatment in medium with 0,1% BSA. (B) Absolute values of serotonin secretion expressed as percentage of untreated control after three days of treatment. (C) Absolute values of CgA secretion expressed as percentage of untreated control after three days of treatment. Asterisks: **,p<0.01; ***,p<0.001, compared to untreated controls.

Chapter 6

Effects of novel somatostatin-dopamine chimeric drugs in 2D and 3D spheroid cell culture models of neuroendocrine tumors

Aura D Herrera-Martínez[1,2,3], Rosanna van den Dungen[1], Fadime Dogan-Oruc[1], Peter M. van Koetsveld[1], Michael D Culler[4], Wouter W. de Herder[1], Raúl M. Luque[2,3] , Richard A. Feelders[1], Leo J. Hofland[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2]Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); Córdoba, Spain.
[3]Reina Sofia University Hospital; Córdoba, Spain.
[4]Ipsen Bioscience; Cambrige, Massachussetts, USA.

Submitted for publication

ABSTRACT

Control of symptoms related to hormonal hypersecretion by functioning neuroendocrine tumors (NETs) is challenging. New therapeutic options are required. Since novel *in vitro* tumor models seem to better mimic the tumor *in vivo* conditions, we aimed to study the effect of somatostatin and dopamine receptor agonists (octreotide and cabergoline, respectively) and novel somatostatin-dopamine chimeric multi-receptor drugs (BIM-065, BIM-23A760) using 2D (monolayer) and 3D (spheroids) cultures.

Methods: Dose-response studies in 2D and 3D human pancreatic NET cell cultures (BON-1 and QGP-1) were performed under serum-containing and serum-deprived conditions. Cell proliferation, somatostatin and dopamine receptors expression (SSTs and D2R), apoptosis, lactate dehydrogenase, as well as serotonin and chromogranin-A (CgA) release were assessed.

Results: 3D-cultures of BON-1/QGP-1 allowed better cell survival than 2D-cultures in serum-deprived conditions. SSTs and D2R mRNA levels were higher in the 3D-model vs. 2D-model. Octreotide/cabergoline/BIM-065/BIM-23A760 treatment did not affect cell-growth or spheroid size. In BON-1 2D-cultures, only BIM-23A760 significantly inhibited CgA release, being this effect more pronounced in 3D-cultures. In BON-1 2D-cultures, cabergoline/BIM-065/BIM-23A760 treatment decreased serotonin release (maximal effect up to 40%), being this effect again more potent in 3D-cultures (up to 67% inhibition; with BIM-23A760 having the most potent effects). In QGP-1, cabergoline/BIM-065 treatment decreased serotonin release only in the 3D-model.

Conclusions: Cultures of NET 3D-spheroids represent a promising method for evaluating cell-proliferation and secretion in NET cell-line models. Compared to 2D-models, 3D-models grow relatively serum-independent. In 3D-model, SSTs-D2R multi-receptor targeting drugs inhibit CgA and serotonin secretion, but not NET cell-growth.

INTRODUCTION

Neuroendocrine tumors (NETs) are rare, usually slow-growing, heterogeneous tumors that originate from the diffuse neuroendocrine system in the gastrointestinal and respiratory tract. Their incidence has increased in the last years, but better prognosis and encouraging increased survival has been reported due to advances in the diagnostic and therapeutic options [1]. Despite this, treatment of NETs remains challenging. Tumor heterogeneity and the fact that a high proportion of patients present with metastatic disease at diagnosis may contribute to it.

The treatment aim of NETs is twofold, i.e. symptom control by inhibition of hormonal overproduction in case of functional NETs and stabilization of tumor growth [2-4]. Somatostatin receptors (SSTs) are widely expressed in NETs, especially the SST_2 and SST_5 subtypes [5-7], where they exert different functions [3]. Somatostatin analogs (SSAs) represent the first therapeutic option in non-resectable well-differentiated NETs, especially in functioning tumors [8], where they exert antitumor activity/tumor stabilization and symptoms control [9-11]. Classical SSAs bind mainly to SST_2 receptors. However, it has been observed that SSAs that bind multiple SSTs-subtypes have increased efficacy in inhibiting growth hormone overproduction, especially when used in combination with dopamine agonists (DAs) [12, 13]. As such, there is increasing interest in multiple receptor targeting by the use of dopastatins, which are chimeric SSTs/dopamine receptor subtype-2 (D2R) ligands that have high affinity to SST_2, SST_5, and D2R [14]. Since O'Toole et al. demonstrated the co-expression of SST_2 and D2R in GEP-NETs [15], the interest in multi-target directed therapies for NETs has increased. Nevertheless, the functional role of co-targeting SSTs and D2R in NETs remains to be fully established.

Moreover, in the last decades there is increasing interest in the development of better *in vitro* models for studying therapies in cancer models. Cell-based assays represent a simple, fast, and cost-effective tool for drugs discovery and evaluation [16]. Most cell-based assays use traditional two-dimensional (2D) monolayer cell cultures on flat, rigid substrates. 2D monolayer cultures have some limitations, and it has been recognized that the effectiveness of drugs in 2D monolayer *in vitro* culture systems are often not representative for drug effectiveness *in vivo* [16]. This discrepancy may be related to drug metabolism *in vivo* and/or the effects of the immune system. At the same time, 2D models lack the cell-cell contact, tumor-stromal interaction and tumor microenvironment [16, 17]. Therefore, during recent years there is increasing interest in the development of *in vitro* models that better mimic the *in vivo* conditions in patients [17]. Currently different methods have been developed to assemble 3D cell culture models. One of these, spheroid cultures, seem to appropriately reproduce tumor cells environment including, cell-cell signaling, growth kinetics, extracellular matrix deposition, nutrients-oxygen conditions, gene expression, drug resistance, cell heterogeneity and cell-cell physical interactions [17]. 3D models with spheroids also allow the evaluation of long-term

treatment and the generation of new treatment models [18-21]. Moreover, such models seems to be a reliable method for evaluating treatment in NETs cell lines [22].
Based on the information described above, the aim of the current study was two-fold: 1) to compare 2D and 3D NET cell culture models in terms of growth and hormone secretion, 2) to evaluate and compare the effects of two chimeric SSTs-D2R multi-receptor targeting drugs (BIM-065 and BIM-760) with the effects of the classical SST_2 and D2R targeting drugs octreotide (OCT) and cabergoline (CAB), respectively, on cell growth and serotonin and chromogranin-A (CgA) secretion in both 2D and 3D pancreatic neuroendocrine tumor (PNET) culture models.

MATERIALS AND METHODS

Cell cultures

We used the human PNET cell lines BON-1 and QGP-1. The BON-1 cell line (kind gift from Dr. Townsend, The University of Texas Medical Branch, Galveston, Tex., USA) was derived from a lymph node metastasis of a human non-functional PNET. The QGP-1 cell line was established from a somatostatin-secreting pancreatic islet cell carcinoma and purchased from the Japanese Collection of Research Bioresources Cell Bank (JRCB, Osaka, Japan) [23].
BON-1 cells were cultured in D-MEM/F12 (GIBCO Biocult Europe, Breda, The Netherlands) containing 10% fetal calf serum (FCS), L-glutamine, fungizone (0.5 mg/L) and penicillin (10^5U/L) (Bristol-Myers Squibb, Woerden, The Netherlands). QGP-1 cells were cultured in RPMI-1640 (GIBCO Biocult Europe, Breda, The Netherlands) containing 10% FCS and penicillin (10^5U/L) (Bristol-Myers Squibb, Woerden, The Netherlands). Cell lines were cultured in 75cm^2 flasks (Greiner bio-one, The Netherlands) at 37°C in a 5% CO_2 incubator. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 85%. Experiments were performed in optimal nutrient conditions (medium containing 10% FCS), as well as in serum-deprived conditions [medium containing 0.1% bovine serum albumin (BSA)].

2D Cultures (monolayer)

Cells were plated in 24-well plates with 1 ml medium at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment (50000 cells/well for BON-1 cells, 30000 cells/well for QGP-1 cells). Treatment was added after 24 hours of incubation (day 0). For the serotonin and CgA assays, cells were cultured in medium containing 0.1% BSA. Cells were incubated for 24 hours in medium with 10% FCS to allow cell attachment, after which the medium was refreshed with medium containing 0.1% BSA. In those experiments performed in 0.1% BSA, a higher number of cells was plated (85000

cells/well for BON-1 cells, 100000 cells/well for QGP-1 cells), again in order to obtain 65–70% cell confluence in the control groups at the end of the experiment.

3D Cultures (spheroids)

Non-scaffold based 3D cell cultures were prepared. Cells were plated in cell-repellent-coated 24-wells plates, (Cellstar®, Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) using a density of 750 cells/well and 1500 cells /well for BON-1 and QGP-1, respectively. After an initial 72 hours of incubation in 1 mL medium with 10% FCS, spheroids were washed two times with medium containing 0.1% BSA, followed by incubation with the indicated drugs and the indicated incubation times, in medium containing 0.1% BSA. This day was defined as day 0 of incubation. Medium and cells were harvested after three and seven days of incubation. In initial experiments, spheroids were also grown in complete medium containing 10% FCS for 3 and 7 days.

Drugs and reagents

CAB was purchased from Sigma-Aldrich (Cat.no. C-0246; Zwijndrecht, The Netherlands), OCT was purchased from Novartis (Basel, Switzerland), and multi-chimeric drugs (BIM-065, BIM-23A760) were provided by IPSEN (Massachusetts, USA). The binding affinity of BIM-23A760 and BIM-065 is shown in Supplemental Table 1. CAB was diluted in 100% EtOH until a stock solution of 10^{-4}M. BIM-065 and BIM-23A760 were diluted in 0.01 N acetic acid with 0.1% BSA, until a stock solution of 10^{-4}M. Further dilutions were made in culture medium until obtaining the final concentrations tested 10^{-7} - 10^{-11} M.

Cell proliferation assay

Measurement of total DNA content

After the incubation period, monolayer cells (2D-model) and spheroids (3D-model) were harvested for DNA measurement, as a measure of cell number. The procedure for the total DNA measurement has been previously described in detail [24, 25]. Briefly, the cell pellet was treated with 150 µL of ammonia solution (1 mol/l) - Triton X 100 (0.2% v/v). 15 minutes later, sonification was performed (Soniprep 150; amplitude 1400 microns). Thereafter, for 2D-model, 1 ml of assay buffer (100 mM NaCl, 100 mM EDTA, 10 mM Tris; pH 7.0) was added, and 20 µl of the solution was mixed with 200 µL of Hoechst dye H33258 solution (1 µg/ml). The fluorescence was measured with the excitation and emission wavelengths set at 350 - 455 nM and referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma-Aldrich, Zwijndrecht, The Netherlands). For 3D-model, the sensitive Quant-iT™ PicoGreen™ dsDNA assay kit (Thermo Fisher Scientific, The Netherlands) was used following the protocol from the manufacturer. The fluorescence was measured at 485 – 535 nM respectively and referenced to the curve of the assay kit.

Spheroids growth measurement

The growth (spheroid size) tracking of the 3D-model was performed by image analysis of spheroids using a Zeiss Axiovert 200/M-based phase-contrast microscope connected to a digital camera. Photographs of spheroids were measured at days 0, 3 and 7 of incubation. The growth was determined by measuring the cross-sectional area using the ImageJ software. Data (measurement in pixels) were compared to untreated control spheroids.

Quantitative RT-PCR

mRNA expression levels of SSTs subtypes (SST_1, SST_2, SST_3, SST_5) and D2R in BON-1 and QGP-1 monolayer and spheroids was evaluated by quantitative RT-PCR (qPCR). We used a previously described method [26]. In short, poly(A^+) mRNA was isolated using Dynabeads Oligo (Deoxythymidine)$_{25}$ (Dynal AS, Oslo, Norway). The poly(A^+) mRNA was eluted in H_2O (65 °C) twice for 2 min each and used for cDNA synthesis in a Tris buffer [50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM dithiothreitol, and 10 mM $MgCl_2$] with 10 U of ribonuclease inhibitor, 2 U of avian myeloblastosis virus Super Reverse Transcriptase, and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 µl. This mixture was incubated for one hour at 42°C, and the resulting cDNA was diluted 5-fold in 160 µl sterile H_20. The total reaction volume (25 µl) consisted of 10 µl of cDNA and 15 µl of TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ). Primers were used at final concentration of 300 nm and probe at 200 nm. Real-time qPCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche) in the ABI Prism 7700 Sequence Detection System (PerkinElmer, Foster City, CA). After two initial heating steps at 50°C (2 min) and 95°C (10 min), samples were subjected to 40 cycles of denaturation at 95°C (15 sec) and annealing at 60°C (60 sec). All samples were assayed in duplicate. Values were normalized against the expression of hypoxanthine-guanine phosphoribosyl-transferase (*HPRT*; used as housekeeping gene). Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set. To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template. The sequence of used primers and efficiencies are described in Supplemental Table 2. The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta Ct}$[27], after efficiency correction of target and reference gene transcripts (*HPRT*) [28].

Chromogranin A and serotonin secretion assay

The medium of monolayer and spheroids cultures was collected from each well. Ascorbic acid (0.1%) was added into all the samples that were used for the serotonin assay. The commercially available human chromogranin A (CgA) ELISA kit (Epitope diagnostics Inc,

San Diego CA, USA) and the serotonin high sensitive ELISA (IBL international, Hamburg Germany) were used following the instructions of the manufacturer.

Apoptosis- lactate dehydrogenase (LDH) Elisa

Cells were plated in 24-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment. At the end of the experiment, medium and cells were collected. A commercially available ELISA (Cell Death Detection ELISA Plus, Roche, The Netherlands) was used to determine apoptosis (DNA fragmentation) in cell lysates according to the protocol of the manufacturer. Similarly, a ELISA kit was used to measure the LDH (pierce LDH cytotoxicity assay kit, Thermo schientific, The Netherlands), to determine LDH release in the medium.

Statistical analysis

For the statistical analysis, the GraphPad Prism version 5 software was used (GraphPhad Software, San Diego, CA). Comparisons between groups were analyzed by the Kruskal–Wallis test (non-parametric data). Differences were taken to be statistically significant at $p<0.05$. Results are expressed as mean ± S.E.M. and as the percentage of the respective controls. Log transformation was used for calculating the IC50.

RESULTS

Pancreas NET cell line model in 2D (monolayer) and 3D (spheroids) cultures

In the BON-1 2D-model, the DNA content increased 5.8-fold after three days and 10.5-fold after seven days of incubation in 10% FCS. In medium containing 0.1% BSA (serum-deprived conditions), the DNA content increased 4-fold after three days and 4.8-fold after seven days of incubation (Figure 1A). In the BON-1 3D-model in medium with 10% FCS, the DNA content increased in a time-dependent manner after three (2.8 fold) and seven days (4.6-fold); in serum deprived conditions, a comparable increase in cell growth was observed after three (1.9-fold) and seven days (4.1-fold; Figure 1B).

In the QGP-1 2D-model in medium containing 10% FCS, a 4.2- and 6-fold increase in cell growth was observed after three and seven days of incubation, respectively. In medium containing 0.1% BSA, DNA content increased only 2.2- and 2.4-fold after three and seven days, respectively (Figure 1C). In the QGP-1 3D-model, the DNA content increased 2.3- and 4-fold after three and seven days, respectively, in medium with 10% FCS and, 1.9- and 3.8-fold after three and seven days, respectively, in serum-deprived conditions (Figure 1D).

In order to evaluate the attenuated cell proliferation in serum-deprived conditions in the 2D-model, we determined the apoptosis and LDH production in both culture models. In both BON-1 (Figure 2A) and QGP-1 (Figure 2C) 2D-models, apoptotic rate at day 7 was

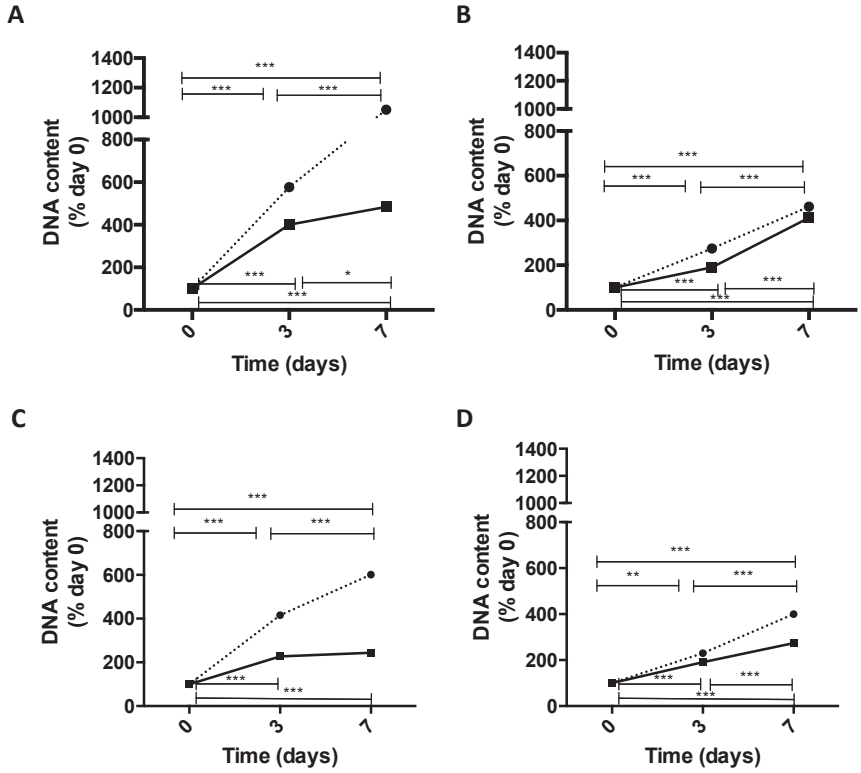


Figure 1: BON-1 and QGP-1 cell growth in 2D- (monolayer) and 3D- (spheroids) cultures in different medium conditions. Cell growth (DNA content per well) was evaluated in untreated control cells at day zero (0) and after three (3) and seven (7) days of incubation. Panels A (2D) and B (3D) cultures of BON-1 cells; panels C (2D) and D (3D) represent cultures of QGP-1 cells. Dotted lines represent cell cultures in medium containing 10% FCS and the continuous black lines represent cell cultures in medium containing 0.1% BSA. Values represent the mean ± SEM and are shown as a percentage of control at day 0. Asterisks: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

7-fold and 1.5-fold higher, respectively, in cells grown in medium with 0.1% BSA compared to cells cultured with 10% FCS medium. However, apoptotic rate was similar or even lower in cells grown in serum-deprived medium, compared to medium with 10% FCS in the BON-1 (Figure 2B) and QGP-1 (Figure 2D) 3D-models.

In serum-deprived medium, but not in medium with 10% FCS, LDH release was detectable in QGP-1 2D-model after 3 and 7 days of incubation and in BON-1 2D-model after 7 days (Supplemental Table 3). On the other hand, LDH was not detectable, even after 7 days of treatment in the 3D-model of both cell lines in serum-deprived and FCS containing medium (data not shown).

In addition, in contrast to the 2D-model, spheroid size and DNA content in BON-1 3D-model strongly increased in a time-dependent manner both in FCS-containing and

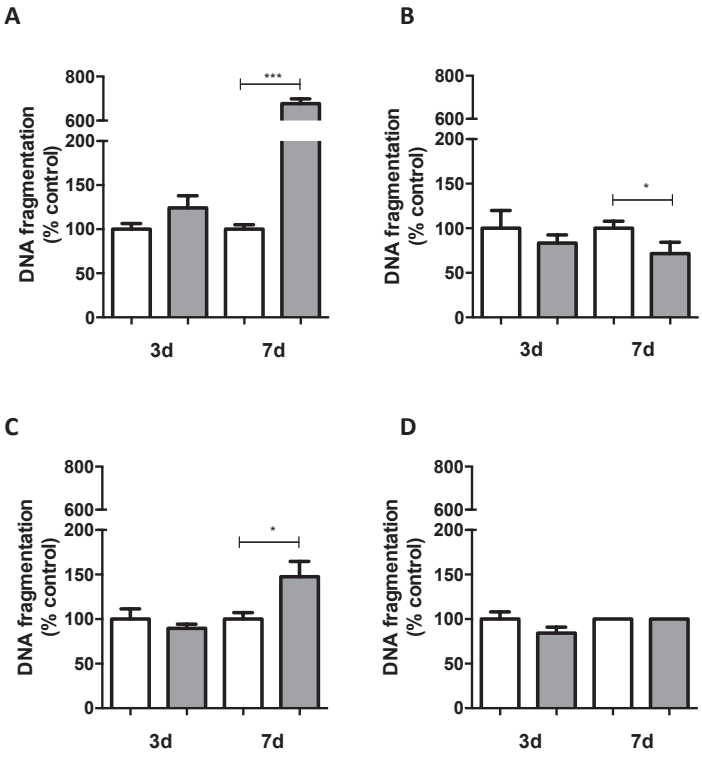


Figure 2: DNA fragmentation (apoptosis) in 2D- and 3D- cultures in BON-1 and QGP-1 cell lines in different medium conditions. Panels A (2D) and B (3D) represent BON-1 cultures; panels C (2D) and D (3D) represent QGP-1 cultures. White bars represent cell cultures in medium containing 10% FCS and the gray bars represent cell cultures in medium containing 0.1% BSA. Apoptosis was higher in BON-1 spheroids after seven days (absolute values of 0.0041±0.0009 - 0.003±0.001 in optimal and serum deprived conditions respectively) than in QGP-1 spheroids (absolute values of 0.001±0 – 0.001±0 in optimal and serum deprived conditions respectively). Values represent the mean ± SEM and are shown as a percentage of cells cultured in 10%FCS. Asterisks: *, p<0.05; ***, p<0.001.

in serum-deprived conditions (Figure 3A, 3B respectively). Similar to what has been observed in BON-1, the size and the DNA content in QGP-1 spheroids increased in a time-dependent manner when cultured in optimal serum conditions (Figure 3C). However, in serum-deprived conditions, the increase in DNA content and spheroid size were dissociated, e.g. DNA content increased in a time-dependent manner, but spheroid size progressively decreased (Figure 3D).

SSTs and D2R expression in monolayer and spheroids cultures

Both BON-1 and QGP-1 cells primarily expressed SST_1, SST_5 and D2R. Overall, the mRNA levels of SSTs subtypes and D2R were considerably higher in BON-1 cells, compared to QGP-1 cells (Supplemental Figure 1). Since the 3D-model may better mimic the

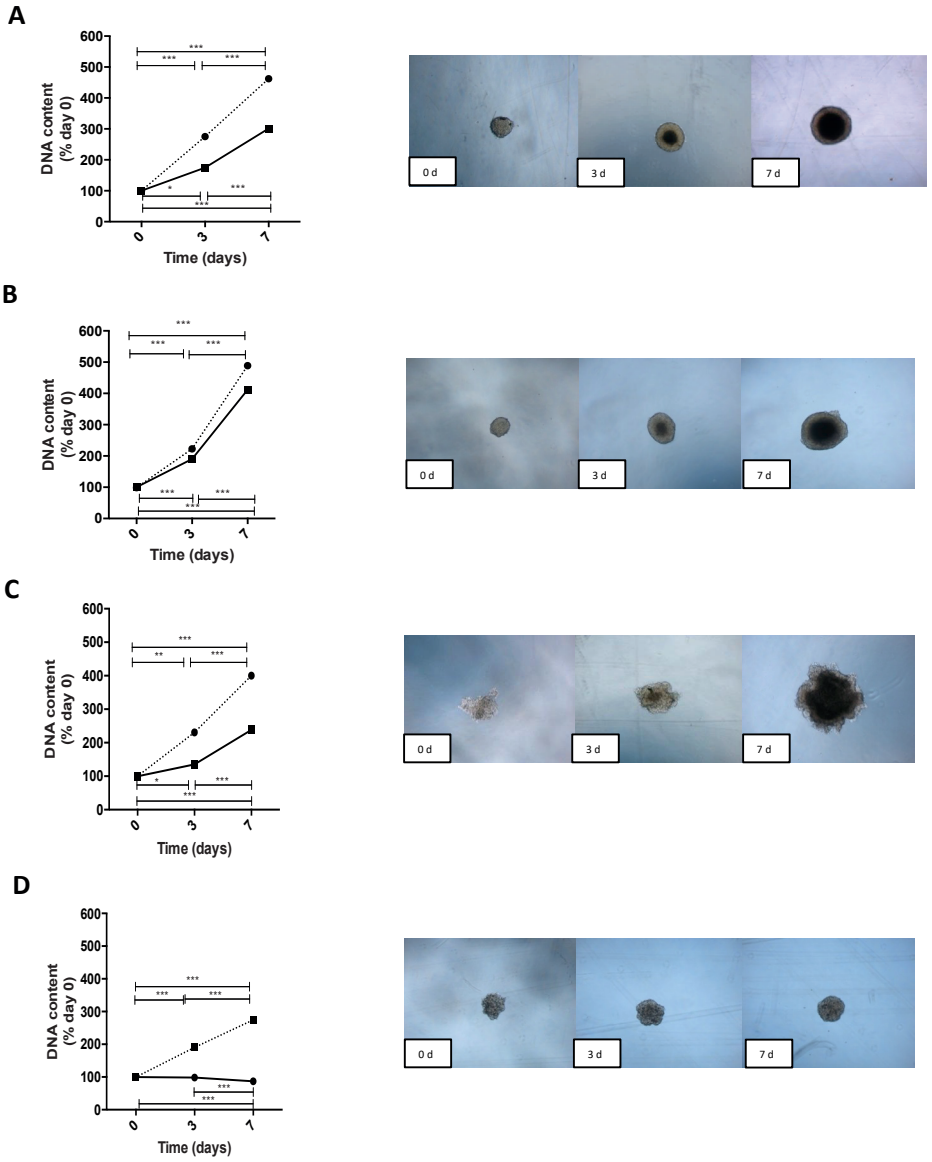


Figure 3: Cell proliferation and spheroid size in BON-1 and QGP-1 cells grown as 3D spheroids in different medium conditions. Cells were followed by imaging using a Zeiss Axiovert 200/M-based phase-contrast microscope using a 5x objective. Representative images of BON-1 and QGP-1 spheroids after their formation (day 0) and after three and seven days culture are depicted in the right panels. Left panels: Increase in BON-1 spheroid cell growth and spheroid size in medium containing 10% FCS (**A**) and 0.1% BSA (**B**); Increase in QGP-1 spheroid cell growth and spheroid size in medium containing 10% FCS (C) and 0.1% BSA (D). Dotted lines represent cell growth (DNA content), continuous black lines represent spheroid size. Values represent mean ± SEM and are shown as a percentage of control at day 0. Asterisks: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

gene expression in solid tumors, we evaluated the time-dependent mRNA levels of SSTs subtypes and D2R in both systems. mRNA levels of SSTs (BON-1 and QGP-1) and D2R (only in QGP-1) were higher in spheroids than in monolayer cultures.
In the 2D-model of BON-1, a time-dependent decrease in the mRNA expression of SST_2 and SST_3 was observed (Figure 4A). In the QGP-1 2D-model, a time-dependent increase in SST_5 expression and a non-significant increase in D2R mRNA levels was observed (Figure 4B).
In the 3D-models, the mRNA levels of SSTs subtypes and D2R was higher in both cell lines compared to the 2D-models of both cell lines. Similar to the BON-1 2D model, mRNA levels of SST_2 decreased and the expression of D2R increased in a time-dependent manner in the spheroid 3D-model (Figure 4C). In QGP-1 spheroids, the mRNA levels of SST_1, SST_2 and SST_5 decreased in a time-dependent manner, whereas the expression of D2R significantly increased (Figure 4D).

Proliferation assays

In BON-1 cells, no statistically significant effect was observed on cell growth (DNA content) after treatment with CAB, BIM-065, BIM-23760 or OCT in 2D-models after three days of incubation in serum-deprived conditions (Figure 5A). Similarly, no effect on cell growth of any of the drugs tested was observed after three and seven days of incubation in medium containing 10% FCS (Supplemental Figures 2A, 2B respectively). In the BON-1 3D-model, also no statistically significant effect was observed on the spheroid growth after three or seven days of treatment in serum-deprived conditions (Figure 5B, 5C respectively). Similar results were observed on QGP-1 2D-model in optimal, FCS containing conditions (Supplemental Figures 2C, 2D), as well as in serum-deprived conditions (Figure 5D) and in spheroid 3D-models (Figure 5E-5F).
In agreement with the absence of an effect of the drugs on cell growth (DNA content), no statistically significant effect was observed on spheroid size after three and seven days of incubation in BON-1 (Supplemental Figures 3A, 3B respectively) or in QGP-1 spheroid 3D-models (Supplemental Figures 3C, 3D respectively).

CgA and serotonin secretion

It was not possible to measure serotonin secretion in BON-1 and QGP-1 cells cultured in medium with FCS, due to a matrix effect in the serotonin assay (data not shown). In order to determine the effect of the drugs on serotonin secretion, PNET cells were cultured in medium containing 0.1% BSA, as described above.
CgA and serotonin secretion was determined in 2D-models only after three days of incubation because of the increased apoptosis and decreased cell viability observed after seven days in serum-deprived medium with 0.1% BSA. In the BON-1 2D-model, BIM-23A760 (10^{-8} – 10^{-10} M) slightly, but statistically significant, inhibited CgA release (11-20%) after three days of treatment (Figure 6A); CgA release was not measurable in QGP-1 cells.

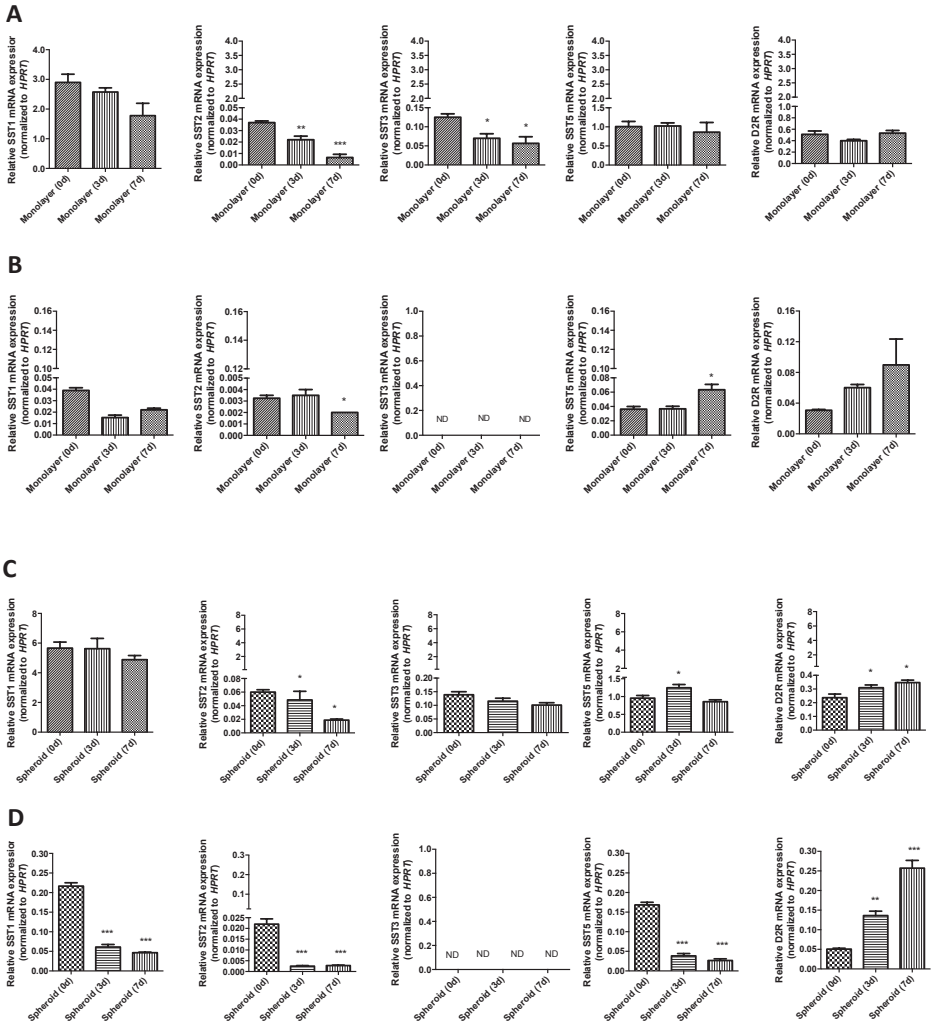


Figure 4: mRNA expression profile of somatostatin receptor subtypes and dopamine type 2 receptor in BON-1 and QGP-1 cell lines using 2D and 3D culture systems. Relative mRNA relative expression (normalized to *HPRT)* in 2D (monolayer) cultures of BON-1 (**A**) and QGP-1 (**B**) cell lines and relative mRNA relative expression in 3D (spheroid) BON-1 (**C**) and QGP-1 (**D**) spheroids. Receptor expression after 3 and 7 days was compared to day 0. **Legend:** ND: not detectable. Asterisks: *, p<0.05; **, p<0.01; ***, p<0.001, compared to monolayer (2D) or spheroid (3D) at day 0.

In the same 2D culture system, serotonin release in BON-1 cells was decreased in a dose-dependent manner by CAB (10^{-7}–10^{-10} M; 14-34%,), BIM-065 (10^{-7}–10^{-11} M; 16-24%) and BIM-23A760 (10^{-7} – 10^{-8} M; 37-43%; Figure 6B). In QGP-1 2D-model, no statistically significant effect was observed on serotonin secretion after three days of incubation with CAB, BIM065, BIM23A760 or OCT (Figure 6C).

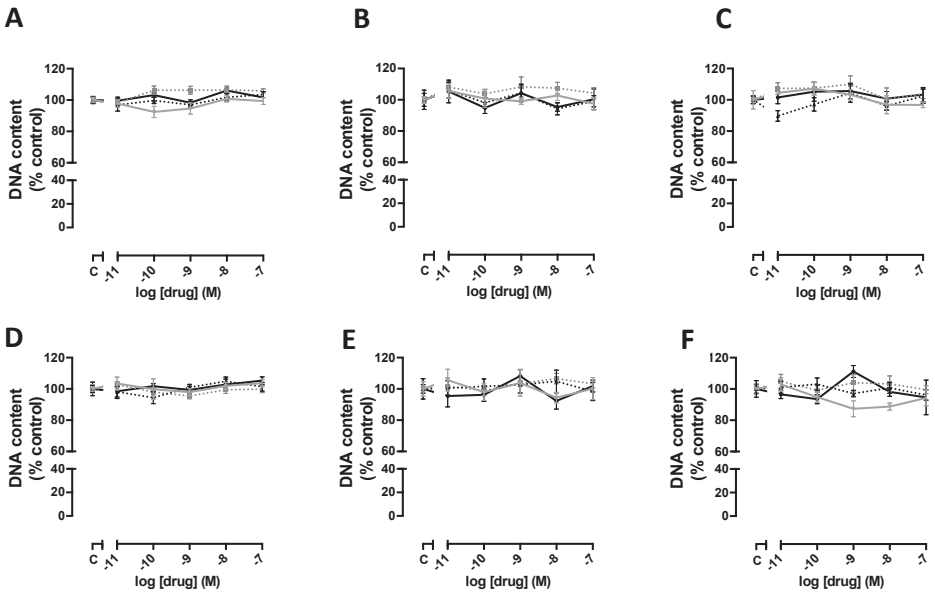


Figure 5: Effect of cabergoline, BIM065, BIM23A760 and octreotide on growth of BON-1 and QGP-1 cells cultured in 2D (monolayer) or 3D (spheroids) in medium with 0.1% BSA. 2D cultures of BON-1 cells after three days of incubation (**A**); 3D cultures of BON-1 cells after three **(B)** or seven **(7)** days of incubation. 2D cultures of QGP-1 cells after three days of incubation (**D**); 3D cultures of QGP-1 cells after three **(E)** or seven **(F)** days of incubation with the indicated drugs. Cell growth (DNA content) is expressed as the percentage of untreated control (mean ± SEM).

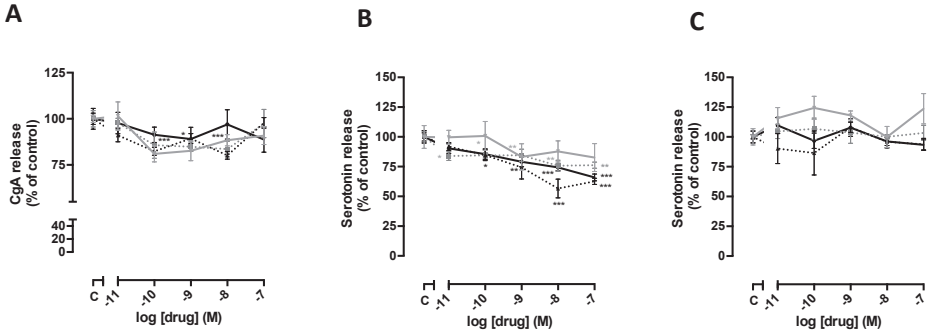


Figure 6: Effect of cabergoline, BIM065, BIM23A760 and octreotide on chromogranin A and serotonin secretion in 2D (monolayer) cultures of BON-1 and QGP-1 cells. Cells were incubated with the indicated drugs during three days in medium with 0.1% BSA. Chromogranin-A (CgA) release in BON-1 cells (**A**); serotonin release in BON-1 cells (**B**); serotonin release in QGP-1 cells (**C**). Values represent mean ± SEM and are shown as a percentage of untreated control. Asterisks: *, p<0.05; **, p<0.01; ***, p<0.001, compared to untreated controls (serotonin absolute values in controls of BON-1: 521.5±57.8; QGP-1: 192.2±16.3 pg/mL; CgA absolute values of BON-1 controls: 721.6±38.8 ng/mL).

CgA and serotonin release in 3D-models were determined only after seven days of incubation because hormone concentrations in the medium after three days of incubation were not high enough (spheroids still too small) to measure accurately. In BON-1 3D-model, CAB (10^{-7} – 10^{-10} M) inhibited CgA release by 18-23% and BIM-065 (10^{-7} – 10^{-10} M) by 31.7-32.3% (Figure 7A). Comparable to the 2D-model, CgA release was not measurable in QGP-1 3D-model. In BON-1 spheroids, serotonin secretion was decreased in a dose-dependent manner by CAB (10^{-7}–10^{-11} M; 36-40%); BIM-065 (10^{-7}–10^{-10} M; 9-42%) and BIM-23A760 (10^{-7}–10^{-11} M; 38-62%; Figure 7B). In QGP-1 spheroids, CAB (10^{-7}–10^{-11} M) and BIM-065 (10^{-7}–10^{-11} M) decreased serotonin production by 29-38% and 25-47%, respectively (Figure 7C).

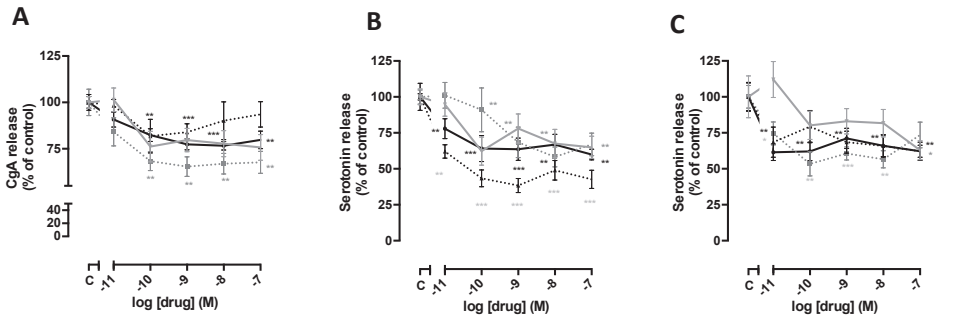


Figure 7: Effect of cabergoline, BIM065, BIM23A760 and octreotide on chromogranin A and serotonin secretion on 3D (spheroid) cultures of BON-1 and QGP-1 cells. 3D spheroids were incubated during seven days with the indicated drugs inmedium with 0.1% BSA. Chromogranin-A (CgA) release in BON-1 cells (**A**); serotonin release in BON-1 cells (**B**); serotonin release in QGP-1 cells (**C**). Values represent mean ± SEM and are shown as a percentage of untreated control. Asterisks: *, p<0.05; **, p<0.01; ***, p<0.001, compared to untreated controls (serotonin absolute values in BON-1 controls: 238.21.5±21.6; QGP-1: 13.67±1.8 pg/mL; CgA absolute values of BON-1 controls: 297.6±14.89 ng/mL).

DISCUSSION

In the present study, we have systematically compared 2D (monolayer) and 3D (spheroid) culture systems of PNET cells in serum-containing and serum-deprived conditions. Since both model systems may provide complimentary information, the effects of SSTs-D2R multi-receptor targeting drugs, SSAs and DAs were evaluated. To the best of our knowledge, this is the first characterization of these PNET cell lines using both culture systems in different nutrient conditions.

In solid tumors, monolayer cell cultures cannot mimic the structure and drug resistance conferred by elements of the tumor microenvironment [29]. This situation may explain the eventual lack of correspondence between some *in vitro* findings and their application in *in*

vivo conditions [17]. In this context, 3D models seem to be a promising method to evaluate anticancer therapies, since they are able to mimic the 3D structure of the tumor and the surrounding microenvironment [29]. These characteristics may explain, for example, the lower apoptotic rate and increased drug resistance found in breast cancer spheroids when compared to monolayer cultures [30]. In our study, a significant lower apoptotic rate was also observed in spheroids when compared to monolayer cultures.

Spheroids also seem to appropriately mimic nutrients availability and hypoxia [31]. Cells located in the peripheral spheroid-area have higher proliferation rates compared to those senescent or necrotic cells located in the center of the spheroid [31]. A difference in the proliferation rate is explained by their easier access to oxygen and nutrients [32] and may be graphically represented with the difference in color intensity inside the BON-1 spheroids, which progressively increased after three/seven days of incubation. According to our results, spheroid cultures of BON-1/QGP-1 cells allowed better cell survival in serum-deprived conditions, admitting longer incubation periods with optimal (time-dependent) increase in the DNA content, even in serum-deprived conditions. This may be useful for evaluating long-term drug effects. The complete lack of correspondence between the DNA content and the spheroid size in QGP-1 3D-model in serum-deprived conditions, suggests that nutrient conditions may influence cell compactness and, as a consequence, the spheroid size. These data emphasize that spheroid size is not always a reliable measure of spheroid cell growth and we strongly advocate that spheroid size in 3D culture models should be correlated with other tumor growth markers before drawing conclusions, especially about drug effectiveness. Similar to our results, previous studies have suggested that spherical (round) spheroids had decreased viability when compared to non-spherical (asymetric) spheroids, probably due to an increased distance between each tumor cell and the culture medium interface [30]. Despite that the apoptosis was higher in BON-1 than in QGP-1 spheroids, apoptosis rate was significantly lower in the spheroid culture system than in the monolayer cultures after seven days of incubation, especially in serum-deprived conditions. Due to the increased apoptotic rate in monolayer cells cultured in serum-deprived conditions after seven days, hormone secretion was only evaluated after three days of incubation. Serotonin secretion in 3D spheroids was lower than in monolayer cultures, due to lower cell numbers. After three days serotonin concentrations were below the detection limit of the assay. For this reason, serotonin production in the spheroid model was evaluated only after seven days.

Previous studies have reported changes in the gene expression of spheroids when compared to monolayer cultures [33, 34]. In this sense, the up-or down-regulation of several genes may explain the differences in drug response in both culture models and may be associated to tumor treatment-resistance [33]. Interestingly, in our study, mRNA levels of SSTs (BON-1 and QGP-1) and D2R (only in QGP-1) were higher in spheroids than in monolayer cultures, which could explain the increased effect of the evaluated drugs in the 3D culture system. Both culture systems exhibited comparable changes in mRNA levels during cultur-

ing, e.g. a time-dependent decreased expression of SST_2 and increased expression of D2R. To the best of our knowledge, time-dependent changes in expression of SSTs and D2R in NET cells has not been reported before, but should be considered when interpreting data from *in vitro* studies. Importantly, both monolayer and spheroid cultures showed the same trend of changes, suggesting this is likely not an explanation for differences between the effects of the SSTs and D2R targeting drugs between the two culture models. Additionally, the increased expression of D2R in spheroids in both cell lines may also explain the potent effect of D2R targeting compounds in this culture system. As described above, NETs express both SSTs and D2R. If our results can be extrapolated to PNETs in situ, our data suggest that SSTs and D2R expression (and possibly response to SSA and D2R agonists) may change during disease progression. It also suggests that the detection of expression level of SSTs and D2R in NET may only be a momentum.

Cell cultures using spheroids have been used to assemble models of different cancer types including breast, cervical, colon, prostate, lung, pancreas and melanoma [35-39]. The spheroid culture system was initially described in BON-1 and QGP-1 cell lines in 2012, and at that moment, the histone-deacetylase inhibitor -Trichostatin A was chosen as the model drug for evaluating 3D cultures in NETs [22]. Recently, the effects of inhibitors of poly(ADP-ribose) polymerase-1 have been also evaluated in a NET 3D culture model [40]. Despite the advantages of 3D cultures for the evaluation of drug effects in cancer, no other treatments have been evaluated in NETs.

It is well known that SSA and dopamine agonists are important therapeutic options in the management of several endocrine tumors, especially in those with hormone overproduction [12]. SSTs and D2R share 30% of their gene sequence [41]. Both family of receptors have been described in NETs [42] and a potential functional role of somatostatin-dopamine receptor dimerization has been also reported [43, 44]. The antiproliferative effect of octreotide and cabergoline has been reported *in vitro* in several types of cancer [45-48] but, similarly to other *in vitro* publications [49] decreased proliferation rate was not observed in our pancreas NET model. The ideal chimeric molecule would have potent SST_2 and D2R affinity, with moderate activity at SST_5 [43]. The antiproliferative effect of somatostatin-dopamine chimeras on cell proliferation seems to be more efficient than SSAs or DAs in enterochromaffin-like cells according to some studies [50], but other authors have described a lower effect on cell proliferation compared to specific SSA in atypical lung carcinoids [51]. It has been suggested that BIM-065 may control tumor cell growth in pituitary adenomas [52], but its effect on cell proliferation in NETs has not been described yet. According to our results, this novel SSTs-D2R chimera does not significantly affect cell proliferation in pancreas NET cell lines. Kidd et al. described a cytotoxic effect of some other somatostatin-dopamine chimeras, including BIM-23A760, in neuroendocrine tumor cells [small intestine, typical and atypical lung carcinoid cell lines [51]]. In contrast, we did not observe an antiproliferative effect of BIM-23A760, which may be explained by the different somatostatin and dopamine

expression profile of these cell lines, when compared to BON-1 and QGP-1 cells. These findings suggest the need to individualize therapeutic strategies based upon the analysis of the receptor profile of the tumor.

In addition, it has been suggested that targeting the D2R receptor is an effective mechanism for suppressing hormone secretion by NET [51]. CAB represents a useful therapeutic tool in the treatment of other hormone-related syndromes [53-55], including pancreatic polypeptide and ACTH-producing NETs [56, 57]. Additionally, somatostatin-dopamine chimeric molecules have shown short-term effectiveness on hormone secretion according to preclinical and clinical trials in endocrine tumors [43], whereas octreotide is recognized as a therapeutic option for carcinoid syndrome [58, 59]. In our study, BIM-23A760 was the most effective drug for inhibiting serotonin release in BON-1 monolayer and BON-1/QGP-1 spheroids, followed by CAB. The effect of BIM-065 was higher in QGP-1 than in BON-1 spheroids and comparable to CAB and BIM-23A760. This is the first report on the effects of this compound on serotonin secretion in NETs. Interestingly, while octreotide is widely used for reducing 5-hydroxiindolacetic acid in patients with NETs [60], in our PNET model, octreotide did not significantly decrease serotonin release in BON-1 monolayer and BON-1/QGP-1 spheroid cultures. Previous *in vitro* reports have described the inhibition of serotonin release by octreotide in human midgut carcinoid tumor cells [61]; the tumor cell origin, the SSTs/D2R expression profile and the incubation period may explain the differences with our results. In addition, the effect of both chimeric compounds seems to be comparable to the dopamine agonist, suggesting that their effect in this model may be predominantly mediated by the D2R. Importantly, the study of the molecular expression of SSTs and D2R in NETs may help to identify those patients suitable for treatment [62]

In contrast, CgA secretion was only slightly inhibited by BIM-23A760 in monolayer cultures and by CAB and BIM-065 in spheroids, which may be related to the absence of effect on cell proliferation, since CgA is considered as a marker related to tumor load [63]. Clinical studies have described a dose-dependent reduction of serum CgA with octreotide [64], which was not observed in our pancreas model.

In summary, this study provides a comprehensive comparison of 2D monolayer and 3D spheroid culture systems in a pancreas model for NETs and suggests that spheroids represent a valuable tool for evaluating long-term drug effectiveness, especially in serum-deprived conditions. Compared to 2D models, 3D models grow relatively serum-independent. In the 3D model, SSTs-D2R multi-receptor targeting drugs inhibit CgA and serotonin secretion, but not NET cell growth. These results further highlight the importance of determining the somatostatin/dopamine receptor profile in NETs in order to identify those patients that may benefit of these compounds.

REFERENCES:

1. Dasari, A., et al., *Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States.* JAMA Oncol, 2017.
2. Herrera-Martinez, A.D., et al., *The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features.* Lung Cancer, 2017. **109**: p. 128-136.
3. Herrera-Martinez, A.D., et al., *Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors.* Endocrine, 2018. **59**(2): p. 426-437.
4. Riihimaki, M., et al., *The epidemiology of metastases in neuroendocrine tumors.* Int J Cancer, 2016. **139**(12): p. 2679-2686.
5. Kubota, A., et al., *Identification of somatostatin receptor subtypes and an implication for the efficacy of somatostatin analogue SMS 201-995 in treatment of human endocrine tumors.* J Clin Invest, 1994. **93**(3): p. 1321-5.
6. Nakayama, Y., et al., *Profiling of somatostatin receptor subtype expression by quantitative PCR and correlation with clinicopathological features in pancreatic endocrine tumors.* Pancreas, 2010. **39**(8): p. 1147-54.
7. O'Toole, D., et al., *ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: biochemical markers.* Neuroendocrinology, 2009. **90**(2): p. 194-202.
8. Falconi, M., et al., *ENETS Consensus Guidelines Update for the Management of Patients with Functional Pancreatic Neuroendocrine Tumors and Non-Functional Pancreatic Neuroendocrine Tumors.* Neuroendocrinology, 2016. **103**(2): p. 153-71.
9. Modlin, I.M., et al., *Gastroenteropancreatic neuroendocrine tumours.* Lancet Oncol, 2008. **9**(1): p. 61-72.
10. Veenstra, M.J., et al., *Targeting the somatostatin receptor in pituitary and neuroendocrine tumors.* Expert Opin Ther Targets, 2013. **17**(11): p. 1329-43.
11. Pavel, M., et al., *ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Neoplasms: Systemic Therapy - Biotherapy and Novel Targeted Agents.* Neuroendocrinology, 2017. **105**(3): p. 266-280.
12. Flogstad, A.K., et al., *A comparison of octreotide, bromocriptine, or a combination of both drugs in acromegaly.* J Clin Endocrinol Metab, 1994. **79**(2): p. 461-5.
13. Marzullo, P., et al., *Efficacy of combined treatment with lanreotide and cabergoline in selected therapy-resistant acromegalic patients.* Pituitary, 1999. **1**(2): p. 115-20.
14. Couvelard, A., et al., *Antisecretory Effects of Chimeric Somatostatin/Dopamine Receptor Ligands on Gastroenteropancreatic Neuroendocrine Tumors.* Pancreas, 2017. **46**(5): p. 631-638.
15. O'Toole, D., et al., *The analysis of quantitative expression of somatostatin and dopamine receptors in gastro-entero-pancreatic tumours opens new therapeutic strategies.* Eur J Endocrinol, 2006. **155**(6): p. 849-57.
16. Edmondson, R., et al., *Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors.* Assay Drug Dev Technol, 2014. **12**(4): p. 207-18.
17. Costa, E.C., et al., *3D tumor spheroids: an overview on the tools and techniques used for their analysis.* Biotechnol Adv, 2016. **34**(8): p. 1427-1441.
18. Baker, B.M. and C.S. Chen, *Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues.* J Cell Sci, 2012. **125**(Pt 13): p. 3015-24.

19. Harma, V., et al., *A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses.* PLoS One, 2010. **5**(5): p. e10431.
20. Kimlin, L.C., G. Casagrande, and V.M. Virador, *In vitro three-dimensional (3D) models in cancer research: an update.* Mol Carcinog, 2013. **52**(3): p. 167-82.
21. Kondo, J., et al., *Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer.* Proc Natl Acad Sci U S A, 2011. **108**(15): p. 6235-40.
22. Wong, C., et al., *Human neuroendocrine tumor cell lines as a three-dimensional model for the study of human neuroendocrine tumor therapy.* J Vis Exp, 2012(66): p. e4218.
23. Kaku, M., et al., *Establishment of a carcinoembryonic antigen-producing cell line from human pancreatic carcinoma.* Gan, 1980. **71**(5): p. 596-601.
24. Downs, T.R. and W.W. Wilfinger, *Fluorometric quantification of DNA in cells and tissue.* Anal Biochem, 1983. **131**(2): p. 538-47.
25. Hofland, L.J., P.M. van Koetsveld, and S.W. Lamberts, *Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995.* Eur J Cancer, 1990. **26**(1): p. 37-44.
26. Ferone, D., et al., *Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes.* Am J Physiol Endocrinol Metab, 2002. **283**(5): p. E1056-66.
27. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.
28. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
29. Breslin, S. and L. O'Driscoll, *Three-dimensional cell culture: the missing link in drug discovery.* Drug Discov Today, 2013. **18**(5-6): p. 240-9.
30. Ka I. Au Ieong, C.Y., Chin To Wong, Angelie C. Shui, Tom T. Y. Wu,Ting-Hsuan Chen, Raymond H. W. Lam, *Investigation of drug cocktail effects on cancer cell-spheroids using a microfluidic drug-screening assay.* Micromachines, 2017. **8**: p. 167.
31. Mehta, G., et al., *Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy.* J Control Release, 2012. **164**(2): p. 192-204.
32. Tredan, O., et al., *Drug resistance and the solid tumor microenvironment.* J Natl Cancer Inst, 2007. **99**(19): p. 1441-54.
33. Francia, G., et al., *Gene expression analysis of tumor spheroids reveals a role for suppressed DNA mismatch repair in multicellular resistance to alkylating agents.* Mol Cell Biol, 2004. **24**(15): p. 6837-49.
34. Schmidt, M., et al., *Spheroid-based 3-dimensional culture models: Gene expression and functionality in head and neck cancer.* Oncol Rep, 2016. **35**(4): p. 2431-40.
35. Amann, A., et al., *Development of an innovative 3D cell culture system to study tumour--stroma interactions in non-small cell lung cancer cells.* PLoS One, 2014. **9**(3): p. e92511.
36. Costa, E.C., et al., *Optimization of liquid overlay technique to formulate heterogenic 3D co-cultures models.* Biotechnol Bioeng, 2014. **111**(8): p. 1672-85.
37. Dufau, I., et al., *Multicellular tumor spheroid model to evaluate spatio-temporal dynamics effect of chemotherapeutics: application to the gemcitabine/CHK1 inhibitor combination in pancreatic cancer.* BMC Cancer, 2012. **12**: p. 15.

38. Ludwig, K., E.S. Tse, and J.Y. Wang, *Colon cancer cells adopt an invasive phenotype without mesenchymal transition in 3-D but not 2-D culture upon combined stimulation with EGF and crypt growth factors.* BMC Cancer, 2013. **13**: p. 221.
39. Vorsmann, H., et al., *Development of a human three-dimensional organotypic skin-melanoma spheroid model for in vitro drug testing.* Cell Death Dis, 2013. **4**: p. e719.
40. Samuel Adant, N.P., Rashmi Shah, Girish Shah, Jean-Mathieu Beauregard, *Potentiation of 177Lu-octreotate PRRT by PARP inhibitors in a 3D spheroid model of human-derived neuroendocrine tumor cell lines* J Nucl Med, 2017. **58**(1 243).
41. Alonso-Gordoa, T., J. Capdevila, and E. Grande, *GEP-NETs update: Biotherapy for neuroendocrine tumours.* Eur J Endocrinol, 2015. **172**(1): p. R31-46.
42. van Adrichem, R.C., et al., *Effects of Somatostatin Analogs and Dopamine Agonists on Insulin-Like Growth Factor 2-Induced Insulin Receptor Isoform A Activation by Gastroenteropancreatic Neuroendocrine Tumor Cells.* Neuroendocrinology, 2016. **103**(6): p. 815-25.
43. Culler, M.D., *Somatostatin-dopamine chimeras: a novel approach to treatment of neuroendocrine tumors.* Horm Metab Res, 2011. **43**(12): p. 854-7.
44. Gatto, F. and L.J. Hofland, *The role of somatostatin and dopamine D2 receptors in endocrine tumors.* Endocr Relat Cancer, 2011. **18**(6): p. R233-51.
45. Ayiomamitis, G.D., et al., *Effects of octreotide and insulin on colon cancer cellular proliferation and correlation with hTERT activity.* Oncoscience, 2014. **1**(6): p. 457-67.
46. Lin, S.J., et al., *Pituitary Tumor Suppression by Combination of Cabergoline and Chloroquine.* J Clin Endocrinol Metab, 2017. **102**(10): p. 3692-3703.
47. Liu, H.L., L. Huo, and L. Wang, *Octreotide inhibits proliferation and induces apoptosis of hepatocellular carcinoma cells.* Acta Pharmacol Sin, 2004. **25**(10): p. 1380-6.
48. Zou, Y., et al., *Somatostatin analogues inhibit cancer cell proliferation in an SSTR2-dependent manner via both cytostatic and cytotoxic pathways.* Oncol Rep, 2009. **21**(2): p. 379-86.
49. Exner, S., et al., *Octreotide Does Not Inhibit Proliferation in Five Neuroendocrine Tumor Cell Lines.* Front Endocrinol (Lausanne), 2018. **9**: p. 146.
50. Kidd, M., et al., *A comparison of the effects of gastrin, somatostatin and dopamine receptor ligands on rat gastric enterochromaffin-like cell secretion and proliferation.* Regul Pept, 2007. **143**(1-3): p. 109-17.
51. Kidd, M., et al., *Differential cytotoxicity of novel somatostatin and dopamine chimeric compounds on bronchopulmonary and small intestinal neuroendocrine tumor cell lines.* Cancer, 2008. **113**(4): p. 690-700.
52. Alexandraki K, K.G., *Pharmacological Management of Cushing's Disease.* JSM Thyroid Disord Manag, 2017. **2**(1).
53. Cozzi, R., et al., *Cabergoline in acromegaly: a renewed role for dopamine agonist treatment?* Eur J Endocrinol, 1998. **139**(5): p. 516-21.
54. Godbout, A., et al., *Cabergoline monotherapy in the long-term treatment of Cushing's disease.* Eur J Endocrinol, 2010. **163**(5): p. 709-16.
55. Koch, A., et al., *Effects of the dopamine agonist cabergoline on the pulsatile and TRH-induced secretion of prolactin, LH, and testosterone in male beagle dogs.* Theriogenology, 2006. **65**(8): p. 1666-77.
56. Pathak, R.D., T.H. Tran, and A.L. Burshell, *A case of dopamine agonists inhibiting pancreatic polypeptide secretion from an islet cell tumor.* J Clin Endocrinol Metab, 2004. **89**(2): p. 581-4.
57. Pivonello, R., et al., *Dopamine receptor expression and function in corticotroph ectopic tumors.* J Clin Endocrinol Metab, 2007. **92**(1): p. 65-9.

58. Baldelli, R., et al., *Somatostatin analogs therapy in gastroenteropancreatic neuroendocrine tumors: current aspects and new perspectives.* Front Endocrinol (Lausanne), 2014. **5**: p. 7.
59. Davis, G.R., et al., *Effect of somatostatin infusion on jejunal water and electrolyte transport in a patient with secretory diarrhea due to malignant carcinoid syndrome.* Gastroenterology, 1980. **78**(2): p. 346-9.
60. O'Toole, D., et al., *Treatment of carcinoid syndrome: a prospective crossover evaluation of lanreotide versus octreotide in terms of efficacy, patient acceptability, and tolerance.* Cancer, 2000. **88**(4): p. 770-6.
61. Wangberg, B., et al., *The effect of a somatostatin analogue on the release of hormones from human midgut carcinoid tumour cells.* Br J Cancer, 1991. **64**(1): p. 23-8.
62. Kanakis, G., et al., *Expression of Somatostatin Receptors 1-5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role.* Neuroendocrinology, 2015. **101**(3): p. 211-22.
63. Nobels, F.R., et al., *Chromogranin A as serum marker for neuroendocrine neoplasia: comparison with neuron-specific enolase and the alpha-subunit of glycoprotein hormones.* J Clin Endocrinol Metab, 1997. **82**(8): p. 2622-8.
64. Al-Efraij, K., M.A. Aljama, and H.F. Kennecke, *Association of dose escalation of octreotide long-acting release on clinical symptoms and tumor markers and response among patients with neuroendocrine tumors.* Cancer Med, 2015. **4**(6): p. 864-70.

SUPPLEMENTAL DATA

Supplementary Table 1: BIM065 and BIM23A760 binding affinity (in nM)

Drug	SST_2	SST_5	D2R
BIM23A760	0,03	42	16
BIM065	0,03	0,5	27.2

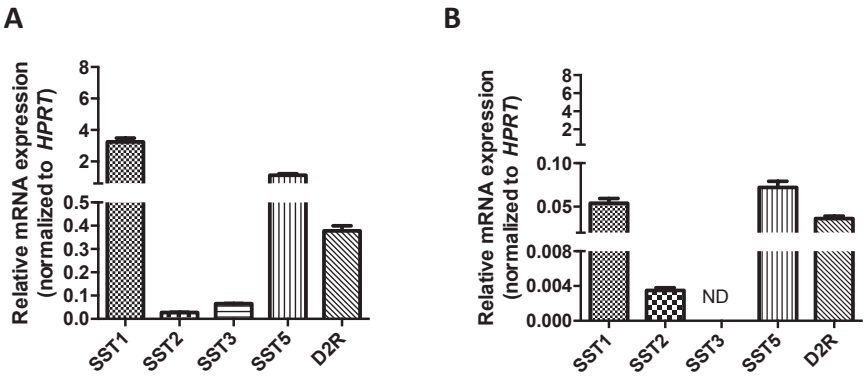
Supplementary Table 2: Primer-probe sequences for SSTR and D2R

Target gene		Sequence	Efficiency
HPRT	Forward	CAC TGG CAA AAC AAT GCA GAC T	1.91
	Reverse	GTC TGG CTT ATA TCC AAC ACT TCG T	
	Probe	CAA GCT TGC GAC CTT GAC CAT CTT TGG A	
SST_1	Forward	CAC CGT GGC CAA GGT AGT AAA	2.00
	Reverse	CCA CGA TGG GCA GGA TGA	
	Probe	CTG GGC GTG TGG GTG CTA TCG C	
SST_2	Forward	TCG GCC AAG TGG AGG AGA C	1.91
	Reverse	AGA GAC TCC CCA CAC AGC CA	
	Probe	CCG GAC GGC CAA GAT GAT CAC C	
SST_3	Forward	CTG GGT AAC TCG CTG GTC ATC TA	1.92
	Reverse	AGC GCC AGG TTG AGG CTG TA	
	Probe	CGG CCA GCC CTT CAG TCA CCA AC	
SST_5	Forward	CAT CCT CTC CTA CGC CAA CAG	1.91
	Reverse	GGA AGC TCT GGC GGA AGT T	
	Probe	CCC GTC CTC TAC GGC TTC CTC TCT GA	
D2R	Forward	GCCACTCAGATGCTCGCC	2.00
	Reverse	ATGTGTGTGATGAAGAAGGGCA	
	Probe	TTGTTCTCGGCGTGTTCATCATCTGC	

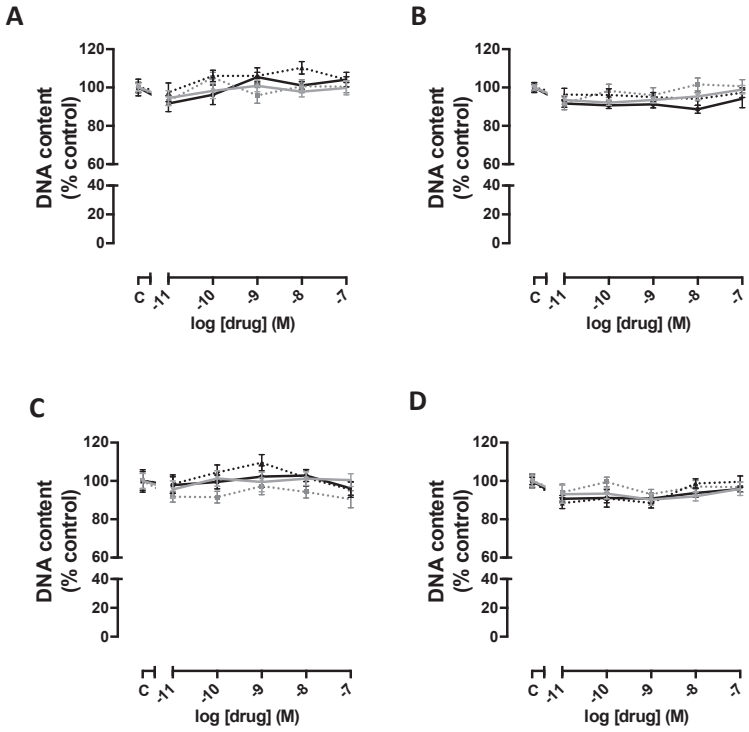
Supplementary Table 3: LDH release in BON-1 and QGP-1 monolayer cultures

	Medium containing 10% FCS		Medium containing 0,1% BSA	
	BON-1 cells	QGP-1 cells	BON-1 cells	QGP-1 cells
3 d	ND	ND	ND	0.02±0.03
7 d	ND	ND	0.82±0.45	0.07±0.03

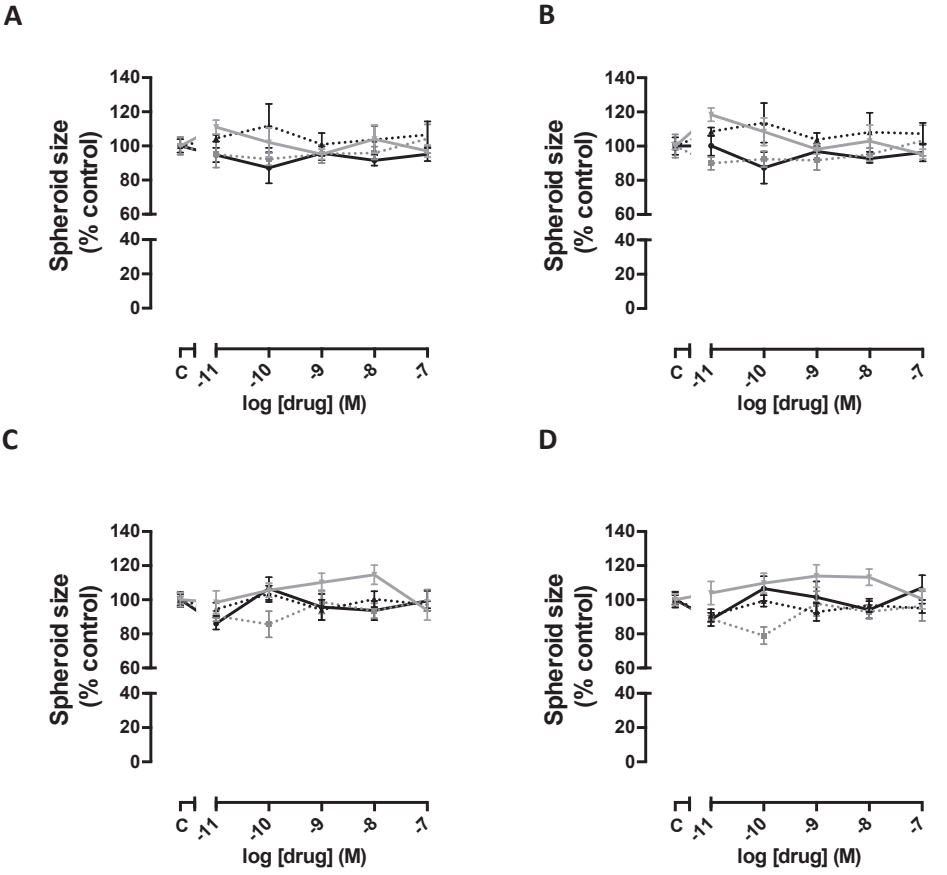
LDH was measured using a spectrophotometer and corrected by DNA Legend: ND: not detectable.



Supplemental Figure 1: mRNA expression profile of somatostatin receptors and dopamine type 2 receptor in BON-1 and QGP-1 cells. Relative mRNA expression (normalized to *HPRT*) in BON-1 (A) and QGP-1 (B) cells. Legend: ND: non-detectable.



Supplemental Figure 2: Effect of CAB, BIM065, BIM23A760 and OCT on cell growth in 2D monolayer cultures of BON-1 and QGP-1 in medium containing 10% FCS. Panels A-B: BON-1 cells after three and seven days of incubation respectively; Panels C-D: QGP-1 cells after three and seven days respectively. Values represent mean ± SEM and are shown as a percentage of untreated control.



Supplemental Figure 3: Effect of cabergoline, BIM065, BIM23A760 and OCT on spheroid size in BON-1 and QGP-1 cells in medium with 0.1% BSA. Change in spheroid size in BON-1 cultures after three (**A**) or seven days (**B**); change in spheroid size in QGP-1 cultures after three (**C**) or seven days (**D**). Values represent mean ± SEM and are shown as a percentage of untreated control.

Chapter 7

Efficacy of the tryptophan hydroxylase inhibitor telotristat on growth and serotonin secretion in 2D and 3D cultured neuroendocrine tumor cells

Aura D Herrera-Martínez[1,2], Richard A. Feelders[1], Rosanna van den Dungen[1], Fadime Dogan-Oruc[1], Peter M. van Koetsveld[1], Justo P. Castaño[2], Wouter W. de Herder[1], Leo J. Hofland[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.

Submitted for publication

ABSTRACT

Serotonin, a biologically active amine, is related to carcinoid syndrome in functioning neuroendocrine tumors (NETs). Telotristat ethyl is a novel inhibitor of the tryptophan hydroxylase (TPH), a key enzyme in the production of serotonin. While its use in patients with carcinoid syndrome and uncontrolled diarrhea under somatostatin analogs (SSAs) has been recently approved, *in vitro* data evaluating it effectiveness are lacking.

For this reason, we aimed to evaluate the effect of telotristat as monotherapy, and in combination with SSAs, on proliferation and secretion in a NET cell line model.

The human pancreatic NET cell lines BON-1/QGP-1 were used as 2D and 3D cultured models; somatostatin receptor and TPH mRNA expression, as well as the potential autocrine effect of serotonin on tumor cell proliferation using a 3D culture system were evaluated.

Telotristat decreased serotonin production in a dose-dependent manner at a clinically feasible concentration, without affecting cell proliferation. Its combination with pasireotide, but not with octreotide, had an additive inhibitory effect on serotonin secretion. The effect of telotristat was slightly less potent, when BON-1 cells were co-treated with octreotide. Octreotide and pasireotide had no effect on the expression of TPH. Telotristat did not have an effect on mRNA expression of somatostatin receptor subtypes. Finally, we showed that serotonin did not have an autocrine effect on NET cell proliferation on the 3D cell model. These results suggest that telotristat is an effective drug for serotonin inhibition, but the effectiveness of its combination with SST_2-preferring SSA should be evaluated more in detail.

INTRODUCTION

Neuroendocrine tumors (NETs) are slow growing neoplasms derived from neoplastic proliferation of enterochromaffin cells, which are able to synthesize, storage and secrete different types of biologically active amines and peptides, including serotonin [1, 2]. Serotonin is synthesized from the essential amino acid l-tryptophan. Tryptophan hydroxylase (TPH) converts tryptophan to 5-hydroxytryptophan, which is subsequently converted to serotonin [3]. Usually serotonin is metabolized by the liver and does not cause systemic symptoms, but when liver metastases are present, many patients present with systemic features of the carcinoid syndrome, including flushing (94%), diarrhea (78%), abdominal pain (51%) and cardiac valvular complications (53%), the latter which can lead to heart failure [4, 5]. Carcinoid syndrome is usually related to metastasized midgut NETs but it may be also observed in patients with bronchial or ovary carcinoids, even if liver metastasis are absent [6]. Systemic levels of serotonin can be measured by tracking the urinary serotonin metabolite 5-hydroxyindoleacetic acid (u5-HIAA). When elevated u5-HIAA is observed, usually the tumor is widely-spread and associated with severe carcinoid syndrome and carcinoid heart disease [7, 8].

Somatostatin analogs (SSAs) are recognized as the standard of care for patients with carcinoid syndrome [9, 10]. Long-acting preparations of SSAs are especially used, since they improve flushes in 53-75% and diarrhea in 45-80% of cases [9]. Despite the effectiveness of SSAs, loss of response during prolonged treatment has been reported. Tachyphylaxis, downregulation of cell surface somatostatin receptors (SSTs), development of antibodies to SSAs, as well as SSTs gene mutations, have been hypothesized [11, 12].

Telotristat ethyl is a novel TPH inhibitor. This drug acts peripherally due to its elevated molecular weight and acidic moieties, which inhibit it from crossing the blood-brain barrier thus avoiding the inhibition of TPH in the central nervous system [8, 13, 14]. Its metabolite, the hippurate salt of telotristat ethyl, reduced serotonin levels throughout the gastro-intestinal tract in mice and improved clinical symptoms without several adverse effects [14]. Additionally, international, multicenter, blind, clinical studies have reported significant reductions in bowel movements frequency and urinary u5-HIAA in patients with carcinoid syndrome not adequately controlled by SSAs [8, 14, 15]. Telotristat ethyl has recently been approved by the US Food and the Drug Administration and the European Medicine Agency for the treatment of diarrhea in those patients with carcinoid syndrome who are inadequately controlled by SSAs. Telotristat is also considered as a category 2A recommendation in the National Comprehensive Cancer Network clinical practice guidelines [16-18]. Despite the promising results reported in clinical trials, surprisingly very little information on the *in vitro* effects of the drug is available in the literature. In this context, we aimed to evaluate the *in vitro* effect of telotristat as monotherapy, as well as in combination with SSAs, on proliferation and secretion in cell line models of NET. To the best of our knowledge, this study extensively

describes for the first time the direct effects of telotristat in two-dimensional (2D) and three-dimensional (3D) cell culture models of NETs.

MATERIALS AND METHODS

Cell cultures

The human pancreatic neuroendocrine tumor cell lines BON-1 and QGP-1 were used. BON-1 cell line (kind gift of Dr. Townsend from the University of Texas Medical Branch, Galveston, Tex., USA) was derived from a lymph node metastasis of a human functioning PNET. The cell line QGP-1 was established from a somatostatin secreting pancreatic islet cell carcinoma and purchased from the Japanese Collection of Research Bioresources Cell Bank (JRCB, Osaka, Japan) [19].

BON-1 cells were cultured in D-MEM/F12 (GIBCO Biocult Europe, Breda, The Netherlands) containing 10% fetal calf serum (FCS), L-glutamine, fungizone (0.5 mg/L) and penicillin 10^5U/L (Bristol-Myers Squibb, Woerden, The Netherlands). QGP-1 cells were cultured in RPMI 1640 (GIBCO Biocult Europe, Breda, The Netherlands) containing 10% FCS (fetal calf serum) and penicillin 10^5U/L (Bristol-Myers Squibb, Woerden, The Netherlands). Cell lines were cultured in 75cm^2 flasks (Greiner bio-one, The Netherlands) at 37°C in a 5% CO_2 incubator. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 87%. For the serotonin assays, cells were cultured in medium containing 0.1% bovine serum albumin (BSA), after an initial incubation of 24 hours in medium with 10% FCS to allow cell attachment.

2D Cultures (monolayer)

Cells were plated in 24-well plates with 1 ml medium at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment (50000 cells/well for BON-1 and 30000 cells/well for QGP-1; data not shown). Drug treatment was started after 24 hours of incubation. In those experiments performed in 0.1% BSA, a higher number of cells was used (85000 cells/well for BON-1 and 100000 cells/well for QGP-1). Cells were incubated during three days with the indicated drugs. Prolonged periods of incubation in serum-deprived monolayer cultures resulted in a significant loss of cell viability.

3D Cultures (spheroids)

Non-scaffold based 3D cell cultures were used. Cells (750 cells/well for BON-1 and 1500 cells/well for QGP-1) were plated in cell-repellent-coated 24-wells plates (cellstar®, Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). After 72 hours of initial incubation in 1 mL medium with 10% FCS, spheroids were washed twice with medium containing

0.1% BSA. Medium was refreshed and drugs were added. Spheroids were incubated during seven days with the indicated drugs, medium and drugs were refreshed at day 3.

Drugs and reagents

The active metabolite of telotristat ethyl (LP-778902, herein named as telotristat) was purchased from Bio-Connect B.V. (The Netherlands). Pasireotide (PAS) and octreotide (OCT) were obtained from Novartis Pharma (Basel, Switzerland). Telotristat was dissolved in dimethyl sulfoxide (DMSO). The evaluated concentrations were chosen on the basis of literature reports and included the clinically relevant plasma concentration (10^{-8} M) [14]. Vehicle (DMSO 0.4% final concentration) was added into the control wells. The calculated half maximal inhibitory concentration (IC_{50}) for both cells lines was used for the combination experiments with SSAs. The IC_{50} and the maximal evaluated concentration was used for evaluating the autocrine effect of serotonin on cell proliferation in the 3D model with spheroids. PAS and OCT were dissolved in medium until obtaining the final concentrations tested 10^{-8} - 10^{-9} M, corresponding to the clinical relevant concentrations according to the literature [20-22].

Measurement of total DNA content

After the incubation period, cells and spheroids were harvested for DNA measurement, as a measure of cell number. The procedure for the total DNA measurement has been previously described in detail [23, 24]. Briefly, cell pellet was treated with 150 µL of ammonia solution (1 mol/l) - Triton X 100 (0.2% v/v). After 15 minutes, sonification was performed (Soniprep 150; amplitude 1400 microns). Thereafter, in 2D cultures, 1 ml assay buffer (100 mM NaCl, 100 mM EDTA, 10 mM Tris; pH 7.0) was added and 20 µl of the solution was mixed with 200 µL of Hoechst dye H33258 solution (1 µg/ml); fluorescence was measured with the excitation and emission wavelengths set at 350 - 455 nM and referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma-Aldrich, Zwijndrecht, The Netherlands). In 3D cultures (spheroids) the Quant-iT™ PicoGreen™ dsDNA assay kit (Thermo Fisher Scientific, The Netherlands) was used, fluorescence was measured with the excitation and emission wavelengths set at 485 – 535 nM and referenced to the standard curve of the kit.

Quantitative RT-PCR

The mRNA expression of SSTs (SST_1, SST_2, SST_3, SST_5) in monolayer and spheroids in both cell lines was evaluated by quantitative RT-PCR. We used a previously described method [25]. In short, poly(A^+) mRNA was isolated using Dynabeads Oligo (Deoxythymidine)$_{25}$ (Dynal AS, Oslo, Norway). The poly(A^+) mRNA was eluted in H_2O (65 ºC) twice for 2 min each and used for cDNA synthesis in a Tris buffer [50 mm Tris-HCl (pH 8.3), 100 mm KCl, 4 mm dithiothreitol, and 10 mm $MgCl_2$] with 10 U ribonuclease inhibitor, 2 U avian myeloblastosis virus Super Reverse Transcriptase, and 1 mm of each deoxynucleotide triphosphate in a final volume of 40 µl. This mixture was incubated for

one hour at 42°C, and the resulting cDNA was diluted 5-fold in 160 µl sterile H_20. The total reaction volume (25 µl) consisted of 10 µl cDNA and 15 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ). Primers were used at final concentration of 300 nm and probe at 200 nm. Real-time qPCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems, Roche) and the ABI Prism 7700 Sequence Detection System (PerkinElmer, Foster City, CA). After two initial heating steps at 50°C (2 min) and 95°C (10 min), samples were subjected to 40 cycles of denaturation at 95°C (15 sec) and annealing at 60°C (60 sec). All samples were assayed in duplicate. Values were normalized against the expression of the housekeeping gene HPRT. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer-probe set [26]. To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template. The sequence of used SSTs primers and efficiencies are described in Supp. Table 1. Tryptophan hydroxylase-1 (TPH-1) PCR primers (Sigma Aldrich) were designed using the Universal Probe Library of Roche (https://www.roche-applied-science.com) on the basis of the reported mRNA sequences in the National Center for Biotechnology Information database (NCBI, Bethesda MD, USA). The primers and their sequences are Forward TGAGACACAGTTCAGATCCCTTC and Reverse GCGGGACATGACCTAAGAT. For each PCR, a mastermix was prepared on ice, containing per sample: 2 µl cDNA, 5 µl of 2x SensiFAST™ SYBR Green Reaction Mix (Bioline Inc, Taunton, MA, USA) and 0.4 µM of both reverse and forward primers. The PCRs were run on a QuantStudio 7 Flex real time PCR system thermocycler (Applied Biosystems, Foster City, CA, USA). The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta C1}$[27], after efficiency correction of target and reference gene transcripts (*HPRT*)[28].

Serotonin secretion assay

The medium of monolayer and spheroids cultures was collected from each well. Ascorbic acid (0.1%) was added into all the samples that were used for the serotonin assay. The commercially available serotonin high sensitive ELISA (IBL international, Hamburg Germany) was used following the instructions of the manufacturer. Experiments were performed in quadruplicate and repeated twice.

Statistical analysis

For the statistical analysis, the statistical software of GraphPad Prism version 5 (GraphPhad Software, San Diego, CA) was used. Between-group comparisons were made by the Kruskal–Wallis test. Differences were considered be statistically significant at $p<0.05$. Results are expressed as mean ± S.E.M. and percentages unless specified otherwise. Log transformation was used for calculating the IC50.

RESULTS

Effects on cell proliferation and serotonin secretion

Telotristat strongly decreased serotonin secretion in a dose-dependent manner in BON-1 (Figure 1A) and QGP-1 cells (Figure 1B) after three days of incubation. QGP-1 cells were more sensitive to telotristat (IC_{50}: 1.3x10^{-9} M; CI 95% 7.3-2.4x10^{-9} M) than BON-1 (IC_{50}: 3.3x10^{-8} M; IC 95% 1.8 - 6.2 x10^{-8} M). The clinically relevant concentration of telotristat (10^{-8} M) decreased serotonin secretion by 40.1±17.4% in BON-1 and by 72.5±15.2% in QGP-1 cells. Serotonin release was totally suppressed in both cell lines after the incubation with the maximal evaluated dose (10^{-5} M). No statistically significant effect on cell growth (DNA content per well) was observed in cells incubated with telotristat for 3 days in medium containing 0,1% BSA (Figures 1A, 1B) or 10% FCS (Supp. Figure 1).

The combination treatment with telotristat and SSAs (pasireotide and octreotide) had no statistically significant effect on cell growth in both cell lines (Figure 2). In BON-1 cells, octreotide 10^{-8}, but not 10^{-9}M, decreased serotonin secretion by 26.7±19.4%. Remarkably, the effectiveness of the combination treatment of telotristat and octreotide on serotonin release was slightly lower than telotristat (T) alone (T: 55.1 ± 13.1%; T+OCT 10^{-8} M: 36.0 ± 29.4%; T+OCT 10^{-9} M: 30.6 ± 25.5%) in BON-1 cells (Figure 1A). Pasireotide (10^{-8}M) decreased serotonin secretion in BON-1 monolayer cultures more potent than octreotide by

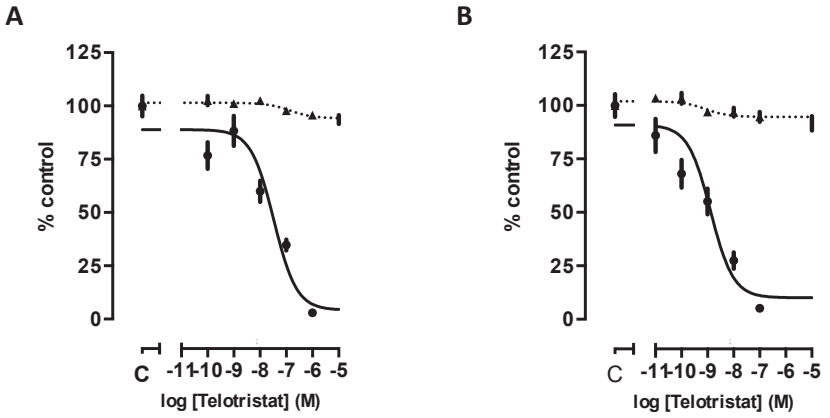


Figure 1: Dose-dependent effect of telotristat on cell growth and serotonin secretion in monolayer PNET cells. (A): Effect of 3 days of treatment with telotristat on cell growth and serotonin secretion in BON-1 cells; (**B):** Effect of 3 days of treatment with telotristat on cell growth and serotonin secretion in QGP-1 cells. The interrupted line represents cell amount, the continuous line represents serotonin secretion. The gray vertical dotted line represents the clinically relevant plasma telotristat concentration. Values represent mean ± SEM and are shown as a percentage of untreated control cell amount (DNA content per well) or serotonin concentration in the culture medium. The mean of serotonin absolute values in BON-1 controls was 1562±130.7 and in QGP-1 cells 84.96±7.9 pg/mL.

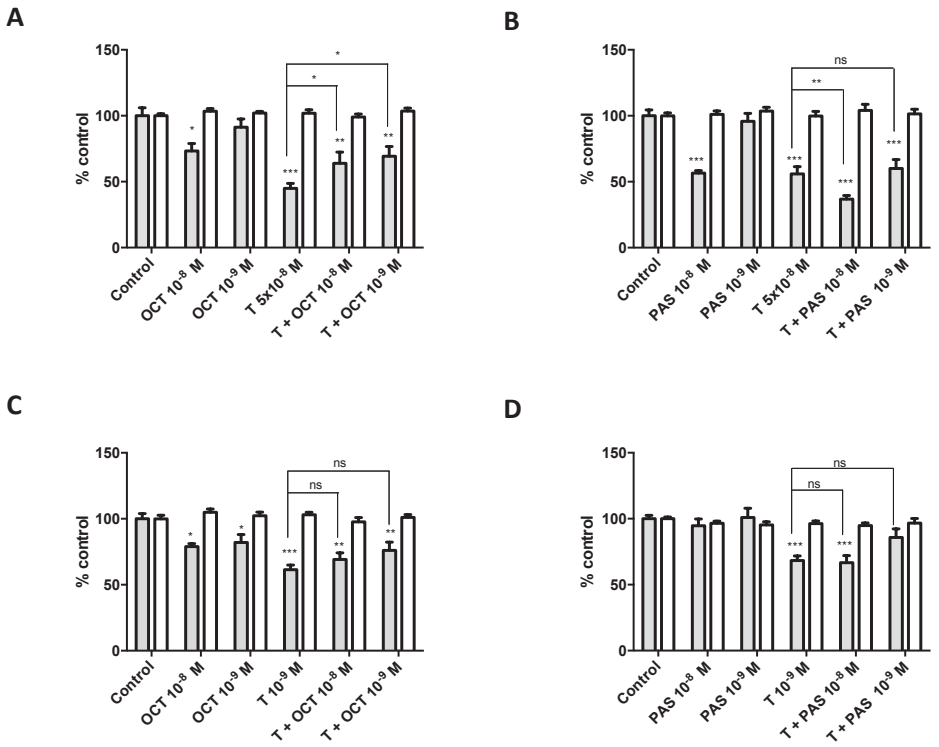


Figure 2: Effect of the combination therapy with telotristat and somatostatin analogs on cell growth and serotonin secretion in monolayer PNET cells. (A) combination treatment of telotristat (T) and octreotide (OCT) in BON-1 cells; **(B)** combination treatment of T and pasireotide (PAS) in BON-1 cells; **(C)** combination treatment of T and OCT in QGP-1 cells; **(D)** combination therapy of T and PAS in QGP-1 cells. For experiments on cell growth, DNA content per well was determined as a measure of cell number. Values represent mean ± SEM and are shown as a percentage of untreated control. Asterisks: *, p<0.05; **, p<0.01; ***, p<0.001, compared to untreated controls. The mean of serotonin absolute values in BON-1 controls was 836.9±67.3 and in QGP-1 cells 285.9±25.4 pg/mL.

43.5 ± 6.6% and its combination with telotristat had an additive effect (serotonin secretion reduction by 63.2 ± 8.9%) when compared to telotristat alone (44.1 ± 19.1; Figure 2B). Pasireotide, at a concentration of 10^{-9}M, did not significantly reduce serotonin secretion in BON-1 cells.

In QGP-1 cells, octreotide 10^{-8} M and 10^{-9} M decreased serotonin secretion by 21.1 ± 7.3% -17.9 ± 21.00 respectively. Combination treatment did not significantly alter the effect of telotristat alone (Figure 2C). Serotonin secretion was not significantly affected by pasireotide (Figure 2D).

Autocrine effect of serotonin on cell proliferation

In order to evaluate the potential autocrine/paracrine *in vitro* effect of serotonin on cell proliferation in BON-1 and QGP-1 cells, a 3D spheroid culture system was used. The reduction in serotonin release in BON-1 spheroids was similar to BON-1 monolayer cultures (Figure 3A). QGP-1 spheroids were slightly less sensitive to the monolayer cultures, but the maximal concentration tested still fully abolished serotonin secretion (10^{-5} M; Figure 3B). Despite the fact that serotonin secretion was totally suppressed after seven days of incubation with the maximal tested dose in both cell lines, no significant changes in spheroid growth (DNA content) were observed in BON-1 (Figure 3A) or QGP-1 3D cultures (Figure 3B). Serotonin levels also did not have an effect on the morphology of the spheroids. Representative images after seven days of incubation without or with telotristat are depicted in Figures 3C (BON-1 spheroids) and 3D (QGP-1 spheroids).

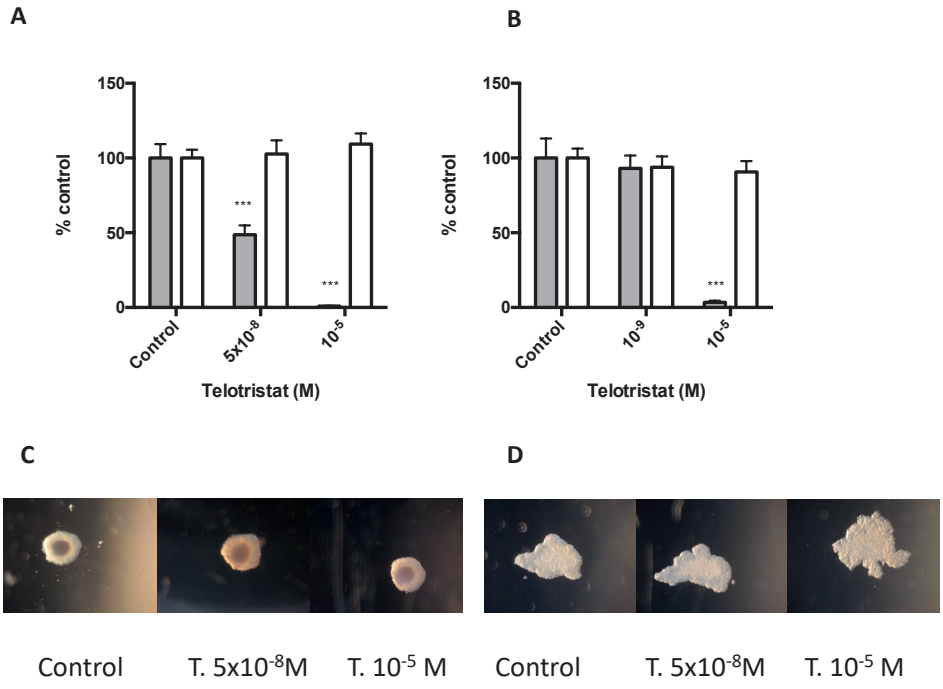


Figure 3: Effect of telotristat on cell growth and serotonin secretion in BON-1 and QGP-1 spheroids. (A) Effect of telotristat on cell growth (DNA content per well) and serotonin secretion in BON-1 spheroids; **(B)** Effects of telotristat on cell growth and serotonin secretion in QGP-1 spheroids; **(C)** representative images of BON-1 and **(D)** QGP-1 spheroids after seven days of incubation with increasing doses of telotristat (T). Values represent mean ± SEM and are shown as a percentage of control. Asterisks: ***, p<0.001. The mean of serotonin absolute values in BON-1 control spheroids was 7555±269.9 and in QGP-1 cells 3363±366.6 pg/mL.

Effect of telotristat on somatostatin receptor expression

In order to rule out the possibility that telotristat influences SSTs expression, we evaluated the effect of telotristat on SSTs subtype expression. Figure shows that telotristat (telotristat $5x10^{-8}$ M for BON-1 and 10^{-9} M for QGP-1) had no significant effect on the expression of SSTs in monolayer cultures of BON-1 and QGP-1 cell lines (Figures 4A and 4B respectively). Similar results were observed in BON-1 and QGP-1 spheroids treated with telotristat (Figures 5A and 5B respectively).

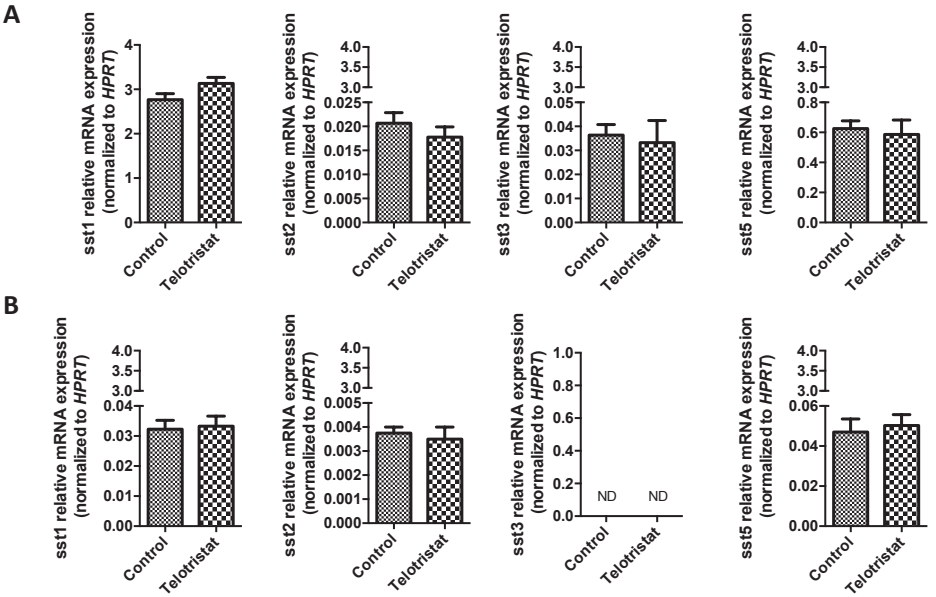


Figure 4: Effect of telotristat on mRNA expression profile of somatostatin receptors in BON-1 and QGP-1 cell lines using monolayer culture systems. Relative mRNA expression normalized to *HPRT* in monolayer cultures of BON-1 (**A**) and QGP-1 (B) cell lines. The mRNA expression of BON-1 and QGP-1 was not statistically significantly altered by telotristat at a concentration of $5x10^{-8}$ and 10^{-9}M, respectively. Values represent mean ± SEM. Legend: ND. Non-detectable

Effect of octreotide and pasireotide on the expression of tryptophan hydroxylase (TPH-1)

In order to explore a putative mechanism of action of telotristat and the interaction with somatostatin analogs, we evaluated the effect of octreotide and pasireotide on TPH-1 expression. Figure shows that octreotide (10^{-8} M) or pasireotide (10^{-8} M) did not have a statistically significant effect on the expression of TPH-1 in monolayer cultures of BON-1 and QGP-1 cell lines (Figures 5A and 5B respectively). In addition, telotristat (telotristat $5x10^{-8}$ M for BON-1 and 10^{-9} M for QGP-1), had no effect on TPH expression.

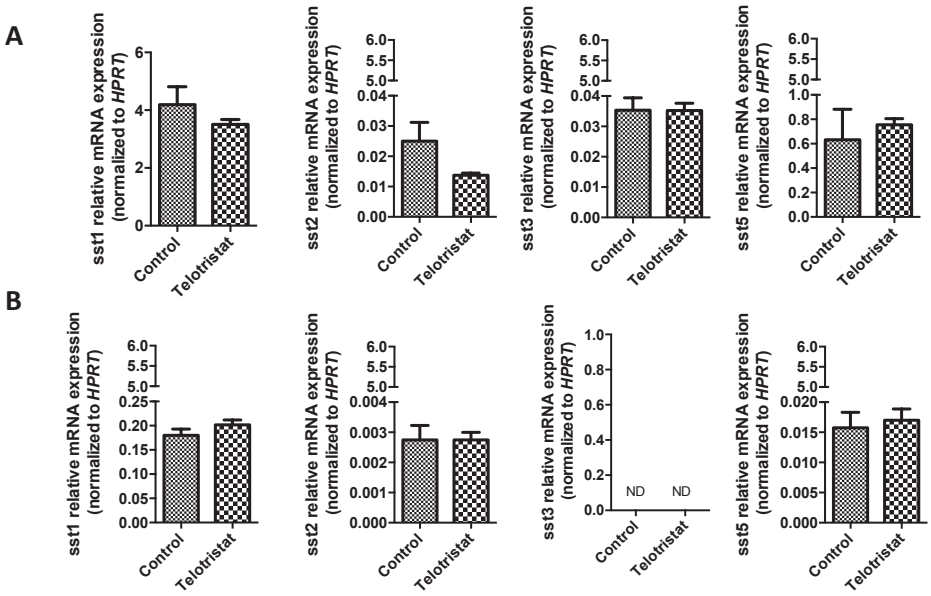


Figure 5: Effect of telotristat on mRNA expression of somatostatin receptors in BON-1 and QGP-1 cell lines using a 3D spheroid culture systems. Relative mRNA expression normalized to *HPRT* in spheroid cultures of BON-1 (**A**) and QGP-1 (B) cell lines. The mRNA expression of BON-1 and QGP-1 spheroids was not statistically significantly altered by telotristat at a concentration of $5x10^{-8}$ and 10^{-9}M, respectively. Values represent mean ± SEM. **Legend:** ND: not-detectable.

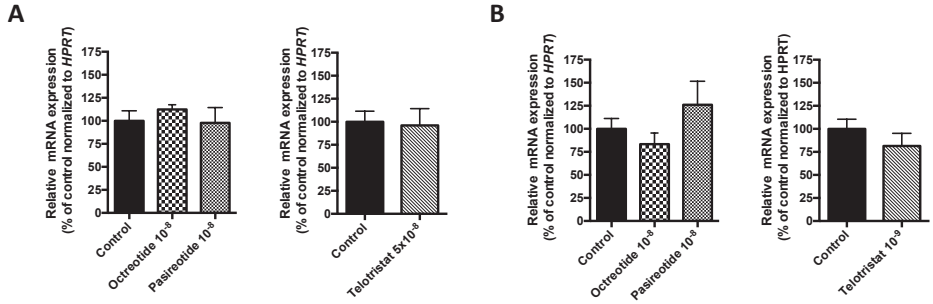


Figure 6: Effect of octreotide, pasireotide and telotristat on mRNA expression profile of tryptophan hydoxylase (TPH-1) in BON-1 and QGP-1 cell lines in monolayer culture. Relative mRNA expression, normalized to *HPRT*, in monolayer cultures of BON-1 (**A**) and QGP-1 (B) cell lines. The mRNA expression of TPH-1 in BON-1 and QGP-1 cells was not statistically significantly altered by octreotide (10^{-8}M), pasireotide (10^{-8}M) or telotristat ($5x10^{-8}$M for BON-1 and 10^{-9}M for QGP-1). Values represent mean ± SEM (relative mRNA expression of TPH-1 in control samples for BON-1: 9.6±1.1; QGP-1: 7.4±0.8).

DISCUSSION

The present study was performed with the aim to evaluate the *in vitro* effects of telotristat and its combination with SSAs on growth and serotonin secretion in a model of pancreatic NET cells. Some publications and clinical trials have reported the clinical and biochemical effects of telotristat in combination with octreotide in patients with carcinoid syndrome [8, 29, 30], and recently its use in patients with carcinoid syndrome and inadequately controlled diarrhea has been approved [16, 17]. Surprisingly, despite the clinical application of telotristat, to the best of our knowledge, only one published study has evaluated the effect of telotristat on synthesis of the serotonin precursor 5-hydroxytryptophan in a pancreatic NET cell line [31]. Therefore, for the first time, we systematically evaluated the *in vitro* effects of telotristat on serotonin secretion and cell growth in mono- and combination-therapy with SSA in two different pancreatic NET cell lines using 2D and 3D cell culture models.

Telotristat did not affect cell proliferation in either monolayer or spheroid cultures, even when very high doses were tested. Moreover, no effects on the morphology of 3D NET spheroids was observed. These results indicate that telotristat does not display any cytotoxicity, even at a very high dose. Previous studies have reported a similar incidence of adverse events in patients treated with telotristat and in those treated with placebo. Adverse events were usually mild or moderate [8, 15, 29]. Elevated liver enzymes have been reported in some patients leading to treatment discontinuation [8, 15], but no serious adverse effects have been described, suggesting the relative safety of use of telotristat in NET patients.

Telotristat strongly decreased serotonin secretion in a dose-dependent manner in both cell lines, in concordance with clinical studies, in which the clinical and biochemical effects of telotristat were shown to be time- and dose-dependent [8, 15, 30]. Moreover, the concentration of telotristat that induced a 50% reduction of serotonin secretion by the NET cell lines ($1.3x10^{-9}$ - $3.3x10^{-8}$M) was in agreement with the clinically relevant concentrations of the drug, i.e. 10^{-8}M[14]. Similarly, preclinical studies have reported decreased intestinal serotonin content after using small-molecule TPH-1 inhibitors [32, 33], and clinical studies also reported statistically significant reduction from baseline u5-HIAA levels in patients with carcinoid syndrome who were already on stable-dose of SSAs during at least three months [8, 15, 30]. Preclinical experimental models of intestinal inflammation have also reported some beneficial roles of TPH1 inhibitors, including reduced colon and jejunum serotonin content, reduced expression of proinflammatory genes and reduced severity of chemical-induced colitis and enteric parasite-induced inflammation in mice [33]. Other benefits have been described after the total blockage of the biosynthesis of gut-derived serotonin by other TPH1 inhibitors, including improvements in the mineral status, microarchitecture and bone strength in animal models with chronic kidney disease [34]. On the other hand, the TPH1 inhibitor LP533401 did not have beneficial effects in an inflammatory model of periodontal disease [35].

Telotristat has been approved for the treatment of carcinoid syndrome (flushes and diarrhea) in combination with SSAs therapy in adults inadequately controlled by SSAs [17]. For this reason, we evaluated the effects of combined treatment with telotristat and SSA. Interestingly, despite the fact that the SST_2 targeting SSA octreotide slightly decreased serotonin secretion in BON-1 cells, its combination with telotristat was slightly less effective than the effect of telotristat alone. In QGP-1, no effect of octreotide on the inhibitory effect of telotristat on serotonin secretion was observed. These results should be evaluated in other *in vitro* or pre-clinical models since they may have application in the clinical practice, not only in terms of costs [36] but also for improving patient treatment. In BON-1, the multiligand SSA pasireotide inhibited serotonin secretion more potent compared to octreotide, which may be explained by the relative high expression of SST_5 in this cell line [37, 38] . Moreover, this inhibitory effect was increased when pasireotide was combined with telotristat. Previous studies have evaluated the applicability of pasireotide in patients with carcinoid tumors resistant to other SSAs [39, 40]. Results in this regard are contradictory, its effectivity and tolerability have been described [39], but other authors have reported that symptom control did not significantly improve [40]. The increased incidence of hyperglycemia may also limit its use as well [41]. It may therefore be important to evaluate the SSTs expression profile of the tumors using RT-qPCR or immunohistochemistry [42-44] in order to consider alternative therapeutic options in functioning NETs.

Additionally, it has been previously described that the inhibition of serotonin may modify the molecular expression of some genes [33]. Since the combination therapy with octreotide slightly reduced the effect of telotristat, we aimed to evaluate the effect of telotristat on mRNA expression of SSTs in monolayer and spheroids. No effect of telotristat on SSTs subtype expression was found, however. Serotonin biosynthesis is regulated by two isoforms of the enzyme tryptophan hydroxylase (TPH) of which TPH-1 is localized predominantly in gastrointestinal enteroendocrine cells and TPH2 in the central nervous system [45]. It is well known that telotristat is a highly specific and potent inhibitor of TPH [14]. In order to further explore the interaction between SSA and telotristat, we evaluated the effect of SSA on TPH-1 expression. However, octreotide and pasireotide did not alter the expression of TPH-1. Therefore, the mechanism of interaction between octreotide and telotristat remains to be elucidated.

Interestingly, BON-1 cells secreted a higher amount of serotonin, compared to QGP-1. Moreover, QGP-1 cells were more sensitive to telotristat than BON-1. Nevertheless, mRNA expression of TPH-1 was comparable between BON-1 and QGP-1 cells, suggesting that other mechanisms responsible for the above differences in serotonin secretion between the two NET cell models.

Finally, several autocrine effects of serotonin have been previously described, especially in relation to tumor progression [46]. Specifically, serotonin may promote the proliferation of several tumor cell types [47-49], including lung non-small cell carcinoma, lung atypical carci-

noid, as well as small intestine NET cells [50]. This autocrine effect may be reversed through the inhibition of serotonin synthesis, release and/or receptor activation [50]. To this aim, we evaluated the paracrine/autocrine effect of serotonin on cell proliferation in a 3D spheroid NET model. This culture system seems to better reproduce tumor cell microenvironment, since it mimics the *in vivo* tumor cell-cell signaling, growth kinetics, extracellular matrix deposition, nutrients-oxygen conditions, gene expression, drug resistance, cell heterogeneity and cell-cell physical interactions [51, 52]. Despite the above earlier observations, we did not observe changes on cell proliferation by treatment with telotristat, even when serotonin production was fully abolished. The discrepancy among the results may be explained by the difference in the origin of the used cell lines, which is also related to the considerable heterogeneity in NETs [53], an also to the amount of serotonin secretion per cell line.

The lack of previously published articles about the direct in vitro effects of telotristat limits the deepness of the discussion of our results, in which we evaluated the effect of telotristat on serotonin producing pancreatic NET cells. The *in vitro* effectiveness of telotristat can be evaluated in a small intestinal (SI) NET cell line model, or even primary cultures of midgut NET, which would represent the ideal tumor model, since carcinoid syndrome is more frequently associated with midgut carcinoids [6, 54]. Midgut NET cell lines are not widely available, however, and appropriate primary cultures difficult to establish. A recent study showed that of seven established NET cell lines, only the SI-NET lines GOT-1, P-STS and the PNET lines BON-1 and QGP-1 displayed a neuroendocrine phenotype and disease-characteristic mutations, while the other supposed SI-NET originating cell lines, e.g. KRJ-I, L-STS and H-STS, did not and were identified as lymphoblastoid (KRJ-1) [55]. Unfortunately, the GOT-1 and P-STS cell lines are not available in our laboratory to confirm our findings in an SI-NET model.

In summary, this study provides for the first time a comprehensive evaluation of the effect of telotristat in a NET model, confirming the potent inhibitory effect of clinically feasible concentrations of telotristat on serotonin release. We provided evidence for the absence of direct cell-toxicity and showed additive inhibitory effects of telotristat and the multiligand SSA pasireotide on serotonin secretion.

REFERENCES:

1. Schnirer, II, J.C. Yao, and J.A. Ajani, *Carcinoid--a comprehensive review.* Acta Oncol, 2003. **42**(7): p. 672-92.
2. de Herder, W.W., *Tumours of the midgut (jejunum, ileum and ascending colon, including carcinoid syndrome).* Best Pract Res Clin Gastroenterol, 2005. **19**(5): p. 705-15.
3. Best, J., H.F. Nijhout, and M. Reed, *Serotonin synthesis, release and reuptake in terminals: a mathematical model.* Theor Biol Med Model, 2010. **7**: p. 34.
4. Rorstad, O., *Prognostic indicators for carcinoid neuroendocrine tumors of the gastrointestinal tract.* J Surg Oncol, 2005. **89**(3): p. 151-60.
5. Kulke, M.H., et al., *Neuroendocrine tumors, version 1.2015.* J Natl Compr Canc Netw, 2015. **13**(1): p. 78-108.
6. van der Lely, A.J. and W.W. de Herder, *Carcinoid syndrome: diagnosis and medical management.* Arq Bras Endocrinol Metabol, 2005. **49**(5): p. 850-60.
7. Zandee, W.T., et al., *Limited value for urinary 5-HIAA excretion as prognostic marker in gastrointestinal neuroendocrine tumours.* Eur J Endocrinol, 2016. **175**(5): p. 361-6.
8. Kulke, M.H., et al., *Telotristat Ethyl, a Tryptophan Hydroxylase Inhibitor for the Treatment of Carcinoid Syndrome.* J Clin Oncol, 2017. **35**(1): p. 14-23.
9. O'Toole, D., et al., *Treatment of carcinoid syndrome: a prospective crossover evaluation of lanreotide versus octreotide in terms of efficacy, patient acceptability, and tolerance.* Cancer, 2000. **88**(4): p. 770-6.
10. Ruszniewski, P., et al., *Rapid and sustained relief from the symptoms of carcinoid syndrome: results from an open 6-month study of the 28-day prolonged-release formulation of lanreotide.* Neuroendocrinology, 2004. **80**(4): p. 244-51.
11. Janson, E.T. and K. Oberg, *Long-term management of the carcinoid syndrome. Treatment with octreotide alone and in combination with alpha-interferon.* Acta Oncol, 1993. **32**(2): p. 225-9.
12. Plockinger, U. and B. Wiedenmann, *Neuroendocrine tumors. Biotherapy.* Best Pract Res Clin Endocrinol Metab, 2007. **21**(1): p. 145-62.
13. Liu, Q., et al., *Discovery and characterization of novel tryptophan hydroxylase inhibitors that selectively inhibit serotonin synthesis in the gastrointestinal tract.* J Pharmacol Exp Ther, 2008. **325**(1): p. 47-55.
14. Lapuerta Pablo, Z.B., Fleming Doug, Wheeler Darren, Sands Arthur, *Telotristat etiprate, a novel inhibitor of serotonin synthesis for the treatment of carcinoid sy ndrome.* Clinical Investigation, 2015. **5**(5): p. 447–456.
15. Pavel, M., et al., *Telotristat ethyl in carcinoid syndrome: safety and efficacy in the TELECAST phase 3 trial.* Endocr Relat Cancer, 2018. **25**(3): p. 309-322.
16. –, E.C.C.i.d.o.g.m.a.u.R.E.N.o.t.E.P.a.o.t.C.f.X.
17. FDA News Release 2017 FDA Approves Xermelo for Carcinoid Syndrome Diarrhea. Silver Springs, M., USA: FDA. (available at: https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm544035.htm).
18. National Comprehensive Cancer Network 2017 Neuroendocrine tumors (version 3.2017). Fort Washington, P., USA: NCCN. (available at: https://www.nccn.org/professionals/physician_gls/pdf/neuroendocrine.pdf).
19. Kaku, M., et al., *Establishment of a carcinoembryonic antigen-producing cell line from human pancreatic carcinoma.* Gan, 1980. **71**(5): p. 596-601.

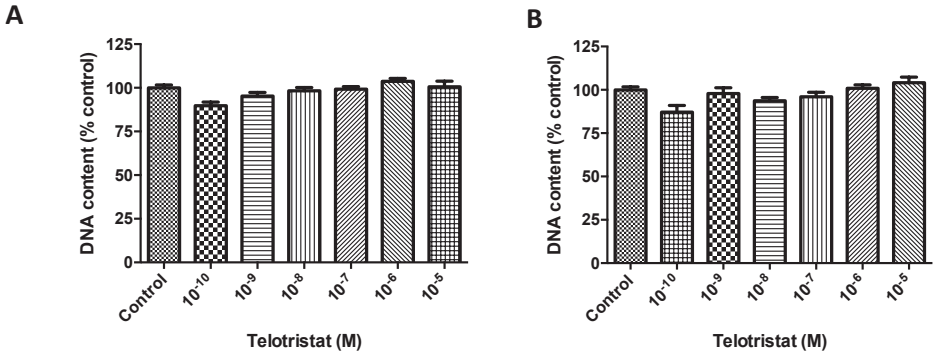
20. Chen, X., et al., *Pharmacokinetics and safety of subcutaneous pasireotide and intramuscular pasireotide long-acting release in Chinese male healthy volunteers: a phase I, single-center, open-label, randomized study.* Clin Ther, 2014. **36**(8): p. 1196-210.
21. Mohamed, A., et al., *Pasireotide and octreotide antiproliferative effects and sst2 trafficking in human pancreatic neuroendocrine tumor cultures.* Endocr Relat Cancer, 2014. **21**(5): p. 691-704.
22. Markham, A., *Telotristat Ethyl: First Global Approval.* Drugs, 2017. **77**(7): p. 793-798.
23. Hofland, L.J., P.M. van Koetsveld, and S.W. Lamberts, *Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995.* Eur J Cancer, 1990. **26**(1): p. 37-44.
24. Downs, T.R. and W.W. Wilfinger, *Fluorometric quantification of DNA in cells and tissue.* Anal Biochem, 1983. **131**(2): p. 538-47.
25. Ferone, D., et al., *Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes.* Am J Physiol Endocrinol Metab, 2002. **283**(5): p. E1056-66.
26. R, R., *Quantification on the LightCycler.* In: Meuer S, Wittwer C and Nakagawara K, eds. Rapid cycle real-time PCR, methods and applications. Heidelberg: Springer Press, 2001: p. 21-34.
27. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.
28. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
29. Kulke, M.H., et al., *Telotristat etiprate, a novel serotonin synthesis inhibitor, in patients with carcinoid syndrome and diarrhea not adequately controlled by octreotide.* Endocr Relat Cancer, 2014. **21**(5): p. 705-14.
30. Pavel, M., et al., *Telotristat etiprate for carcinoid syndrome: a single-arm, multicenter trial.* J Clin Endocrinol Metab, 2015. **100**(4): p. 1511-9.
31. Welford, R.W., et al., *Serotonin biosynthesis as a predictive marker of serotonin pharmacodynamics and disease-induced dysregulation.* Sci Rep, 2016. **6**: p. 30059.
32. Margolis, K.G., et al., *Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine.* Gut, 2014. **63**(6): p. 928-37.
33. Kim, J.J., et al., *Blocking peripheral serotonin synthesis by telotristat etiprate (LX1032/LX1606) reduces severity of both chemical- and infection-induced intestinal inflammation.* Am J Physiol Gastrointest Liver Physiol, 2015. **309**(6): p. G455-65.
34. Pawlak, D., et al., *LP533401 restores bone health in 5/6 nephrectomized rats by a decrease of gut-derived serotonin and regulation of serum phosphate through the inhibition of phosphate co-transporters expression in the kidneys.* Bone, 2018. **113**: p. 124-136.
35. Lima, G.M., et al., *The effect of an inhibitor of gut serotonin (LP533401) during the induction of periodontal disease.* J Periodontal Res, 2016. **51**(5): p. 661-8.
36. Joish, V.N., F. Frech, and P. Lapuerta, *Cost-effectiveness analysis of telotristat ethyl for treatment of carcinoid syndrome diarrhea inadequately controlled with somatostatin analogs.* J Med Econ, 2018. **21**(2): p. 182-188.
37. Herrera-Martínez AD, F.R., Castaño JP, Van Dungen R, Dogan-Oruc F, Van Koetsveld P, Hofland LJ, *Potential Effects of Ketoconazole on ACTHProducing and Non-ACTH-Producing Neuroendocrine Tumors.* ENETS Conference, Abstract C06. Barcelona., 2018.
38. Veenstra, M.J., et al., *Epidrug-induced upregulation of functional somatostatin type 2 receptors in human pancreatic neuroendocrine tumor cells.* Oncotarget, 2018. **9**(19): p. 14791-14802.

39. Kvols, L.K., et al., *Pasireotide (SOM230) shows efficacy and tolerability in the treatment of patients with advanced neuroendocrine tumors refractory or resistant to octreotide LAR: results from a phase II study.* Endocr Relat Cancer, 2012. **19**(5): p. 657-66.
40. Wolin, E.M., et al., *Phase III study of pasireotide long-acting release in patients with metastatic neuroendocrine tumors and carcinoid symptoms refractory to available somatostatin analogues.* Drug Des Devel Ther, 2015. **9**: p. 5075-86.
41. Cives, M., et al., *Phase II clinical trial of pasireotide long-acting repeatable in patients with metastatic neuroendocrine tumors.* Endocr Relat Cancer, 2015. **22**(1): p. 1-9.
42. de Herder, W.W., et al., *Somatostatin receptors in gastroentero-pancreatic neuroendocrine tumours.* Endocr Relat Cancer, 2003. **10**(4): p. 451-8.
43. Kanakis, G., et al., *Expression of Somatostatin Receptors 1-5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role.* Neuroendocrinology, 2015. **101**(3): p. 211-22.
44. Herrera-Martinez, A.D., et al., *Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors.* Endocrine, 2018. **59**(2): p. 426-437.
45. Swami, T. and H.C. Weber, *Updates on the biology of serotonin and tryptophan hydroxylase.* Curr Opin Endocrinol Diabetes Obes, 2018. **25**(1): p. 12-21.
46. Sarrouilhe, D., et al., *Serotonin and cancer: what is the link?* Curr Mol Med, 2015. **15**(1): p. 62-77.
47. Koba, S., et al., *Vascular smooth muscle proliferation: synergistic interaction between serotonin and low density lipoproteins.* J Am Coll Cardiol, 1999. **34**(5): p. 1644-51.
48. Banasr, M., et al., *Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone.* Neuropsychopharmacology, 2004. **29**(3): p. 450-60.
49. Liang, C., et al., *Serotonin promotes the proliferation of serum-deprived hepatocellular carcinoma cells via upregulation of FOXO3a.* Mol Cancer, 2013. **12**: p. 14.
50. Drozdov, I., et al., *Autoregulatory effects of serotonin on proliferation and signaling pathways in lung and small intestine neuroendocrine tumor cell lines.* Cancer, 2009. **115**(21): p. 4934-45.
51. Edmondson, R., et al., *Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors.* Assay Drug Dev Technol, 2014. **12**(4): p. 207-18.
52. Costa, E.C., et al., *3D tumor spheroids: an overview on the tools and techniques used for their analysis.* Biotechnol Adv, 2016. **34**(8): p. 1427-1441.
53. Cives, M., H.P. Soares, and J. Strosberg, *Will clinical heterogeneity of neuroendocrine tumors impact their management in the future? Lessons from recent trials.* Curr Opin Oncol, 2016. **28**(4): p. 359-66.
54. van der Horst-Schrivers, A.N., et al., *Complications of midgut carcinoid tumors and carcinoid syndrome.* Neuroendocrinology, 2004. **80 Suppl 1**: p. 28-32.
55. Hofving, T., et al., *The neuroendocrine phenotype, genomic profile and therapeutic sensitivity of GEPNET cell lines.* Endocr Relat Cancer, 2018. **25**(3): p. 367-380.

SUPPLEMENTAL DATA

Supplementary Table 1: Primer-probe sequences for SSTR and D2R

Target gene		Sequence	Efficiency
HPRT	Forward	CAC TGG CAA AAC AAT GCA GAC T	1.91
	Reverse	GTC TGG CTT ATA TCC AAC ACT TCG T	
	Probe	CAA GCT TGC GAC CTT GAC CAT CTT TGG A	
SST_1	Forward	CAC CGT GGC CAA GGT AGT AAA	2.00
	Reverse	CCA CGA TGG GCA GGA TGA	
	Probe	CTG GGC GTG TGG GTG CTA TCG C	
SST_2	Forward	TCG GCC AAG TGG AGG AGA C	1.91
	Reverse	AGA GAC TCC CCA CAC AGC CA	
	Probe	CCG GAC GGC CAA GAT GAT CAC C	
SST_3	Forward	CTG GGT AAC TCG CTG GTC ATC TA	1.92
	Reverse	AGC GCC AGG TTG AGG CTG TA	
	Probe	CGG CCA GCC CTT CAG TCA CCA AC	
SST_5	Forward	CAT CCT CTC CTA CGC CAA CAG	1.91
	Reverse	GGA AGC TCT GGC GGA AGT T	
	Probe	CCC GTC CTC TAC GGC TTC CTC TCT GA	
D2R	Forward	GCCACTCAGATGCTCGCC	2.00
	Reverse	ATGTGTGTGATGAAGAAGGGCA	
	Probe	TTGTTCTCGGCGTGTTCATCATCTGC	



Supplemental Figure 1: Effect of 3 days of treatment with telotristat on PNET cell growth in medium containing 10% FCS (A): effect of telotristat on cell growth (DNA content per well) in BON-1 cells; **(B):** effect of telotristat on cell growth in QGP-1 cells. Values represent mean ± SEM and are expressed as the percentage of untreated controls.

Chapter 8

Type 2 diabetes in neuroendocrine tumors: are biguanides and statins part of the solution?

Aura D. Herrera-Martínez[1,2], Sergio Pedraza-Arevalo[1,3], Fernando López-López[1,3,4], Manuel D. Gahete[1,3,4], María A. Gálvez-Moreno[1,2], Justo P. Castaño[1,3,4], Raúl M. Luque[1,3,4]

[1]Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); Córdoba, Spain
[2]Endocrinology and Nutrition Service, Reina Sofia University Hospital; Córdoba, Spain
[3]Department of Cell Biology, Physiology, and Immunology, University of Córdoba; Córdoba, Spain

J Clin Endocrinol Metab. 2018 Sep 27. doi: 10.1210/jc.2018-01455.

ABSTRACT

Background: Biguanides and statins have been reported to exert beneficial effects on various cancer types. However, their precise effects and underlying molecular mechanisms are still poorly understood. **Materials and Methods:** We analyzed the relation between metabolic-syndrome, i.e., presence of type-2 diabetes (T2DM), hyperlipidemia and their treatment, with histological, epidemiological, and prognosis variables in two patient cohorts with neuroendocrine-tumors [NETs: lung-carcinoids (LCs; n=81) and gastro-entero-pancreatic (GEP-NET; n=100)]. Additionally, we investigated the antitumoral effects of different biguanides and statins by evaluating proliferation/migration/secretion/gene-expression and involved molecular pathways using BON1/QGP1-cell cultures. **Results:** In T2DM patients, pleura invasion was higher (LCs group; $p<0.05$) and tumor diameter tended to be increased (GEP-NET group). mRNA levels of somatostatin and ghrelin systems were different in tumor tissue of T2DM patients with and without metformin. Biguanides (metformin/buformin/phenformin) decreased proliferation rate in BON1/QGP1-cells (24-72h). However, the effects of statins on proliferation-rate were dependent of the statin-type, cell-type, and time. Specifically, only simvastatin/atorvastatin decreased proliferation in BON1-cells (48/72h and 72h, respectively), while all statins decreased proliferation rate in QGP1-cells (48/72h). Remarkably, metformin and simvastatin decreased migration capacity in BON1-cells and biguanides decreased serotonin secretion in BON1-cells. Phenformin increased apoptosis in BON1/QGP1-cells, and simvastatin in QGP1-cells. These antitumor effects likely involved altered expression of key genes related to cancer aggressiveness (i.e. *GLUT4, INSR*). Altogether, our results reveal a clear inhibitory effect of biguanides and statins on NET-cell aggressiveness. **Conclusion:** Given the demonstrated clinical safety of these drugs, our results invite to further explore their potential therapeutic role for the treatment of NET patients.

INTRODUCTION

Biguanides comprise a class of drugs with relevant effects as insulin-sensitizing agents, which are consequently used to treat type 2 diabetes (T2DM), a severe disease with distinct co-morbidities and whose incidence is growing worldwide, along with its associated metabolic syndrome and their other concomitant diseases (1). The inflammation and insulin resistance present in patients with T2DM or metabolic syndrome have been associated with increased incidence of neoplasms (2); thus, some treatment options targeting related pathways, as may be the case of biguanides, could be beneficial in some types of cancer. In this context, a putative specific relationship between T2DM-metabolic syndrome and neuroendocrine tumors (NETs) has not been established yet.

Among biguanides, only metformin is commercially available for medical use, since it has a safe profile and is well tolerated. Phenformin and buformin were withdrawn in the early 1970s because of an association with lactic acidosis and increased cardiac mortality (3, 4). Interestingly, a putative association between metformin treatment and cancer prevention/treatment was suggested in 2005 (5), and multiple investigations have been subsequently published on this topic. Specifically, some epidemiological studies have suggested a decreased risk for pancreas, liver, colon, lung, and breast cancer in patients with diabetes treated with metformin (6-9). This protective effect of metformin for cancer has been also found in diabetic patients according to several meta-analysis (9-11). Moreover, biguanides can inhibit cell proliferation *in vitro* in several cancer cell lines, including pancreatic and neuroendocrine tumor cells (12, 13). In terms of signaling, biguanides stimulate AMP-activated protein kinase (AMPK), reduce hepatic gluconeogenesis/glycogenolysis and increase glucose uptake in the muscle (14, 15). AMPK activation also suppresses the mammalian target of rapamycin (mTOR1), which is a key regulator of proliferation in cancer cells. AMPK induces cell cycle arrest and reduces the insulin/insulin like growth factor 1 (IGF-1) signaling (16, 17). Metformin-mediated AMPK activation may also result in p53-mediated cell cycle arrest or apoptosis (18, 19). It has been also described that metformin could inhibit cell proliferation by G0/G1, G2/M or S phase arrest (20). However, metformin may also exert antineoplastic properties in an AMPK-independent manner (21).

Statins are also commonly used drugs in the therapeutic arsenal for patients with metabolic syndrome or T2DM. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR, HMGCoAR), affecting the rate limiting step in cholesterol synthesis, but they also exert other clinical effects related with immunomodulatory mechanisms in vascular diseases, autoimmune diseases and organ transplantation (22). In addition, statins also reduce bone marrow stimulation and have been shown to exert anti-proliferative effects on smooth muscle cells (23-26). The antitumor mechanisms of statins may include: induced cell-cycle arrest, apoptosis induction and activation of the signaling of c-Jun N-terminal kinases (JNKs), decreased invasion/metastasis capacity and decreased *MKI67* expression

(27-31). These antitumor effects have been described in several tumor types, including melanoma, colon and breast cancer (30-33). Moreover, statins have been proposed as well as an useful treatment option to induce apoptosis and decrease proliferation in pheocromocytomas and paragangliomas (34, 35); but, to the best of our knowledge, studies with statins have not yet been reported in NETs.

Since the anti-neoplastic therapy in advanced NETs is still unsatisfactory, novel drugs for tumor growth control are required, especially in progressive and hereditary NETs, which are characterized by early onset and multiple lesions (12). Therefore, based on the potential association between T2DM, metabolic syndrome and cancer, we explored this association in a well-characterized cohort of lung carcinoids (LCs) and gastroenteropancreatic neuroendocrine tumors (GEP-NETs). In addition, we analyzed the use of antidiabetic drugs and statins in these cohorts and explored their putative relationship with clinical/histological characteristics. Finally, we also investigated , for the first time, the potential *in vitro* antitumoral effects of different biguanides (i.e. metformin, buformin and phenformin) and statins (i.e. atorvastatin, lovastatin rosuvastatin and simvastatin) in two different NET-cell models, BON1 and QGP1 cell lines.

MATERIALS AND METHODS

Patients and tissue samples

This study was approved by the Ethics Committee of the Reina Sofia University Hospital (Córdoba, Spain), and was conducted in accordance with the Declaration of Helsinki, and according to national and international guidelines. A written informed consent was signed by every individual before inclusion into the study. A total of 181 patients [81 with lung carcinoids (LCs), and 100 with gastroenteropancreatic (GEP-NETs)] who underwent surgery at the Reina Sofia University Hospital from 2005 to 2015 were included in the study. Clinical records were used to collect full medical history. Endocrine-associated syndromes were excluded. Patients with T2DM before the diagnosis of NET (31 patients) were analyzed separately: 14 T2DM patients in the LCs group, 6 treated with metformin; and 17 T2DM patients in the GEP-NETs group, 9 treated with metformin. Similar analysis was performed in those patients treated with statins: 4 in the LCs and 6 in the GEP-NETs group. Demographic and clinical characteristics of both cohorts are summarized in Tables 1-4. Patients were managed following the available guidelines and recommendations. After surgery, if residual or relapsed disease was observed, adjuvant treatment with or without surgery was prescribed. To confirm the neuroendocrine nature of all tumors, chromogranin A, synaptophysin, cytokeratin 7, cytokeratin 20, CD56 and neuronal specific enolase were determined by immunohistochemistry, which was performed following the standardized diagnosis protocol of our hospital and evaluated by two experienced pathologists. Formalin-

fixed paraffin-embedded samples were available in 46 LCs and 55 GEP-NETs cases, in which total RNA was isolated. Tumor samples were re-evaluated by two experienced pathologists before RNA isolation. Only primary tumor samples were included. Samples were analyzed individually and mRNA expression levels were correlated with the clinical/histological characteristics of the corresponding patient.

Culture of cell lines

We used two human pancreatic NET (PNET) cell lines: BON-1 and QGP-1 (36-39). BON-1 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-F12; Life Technologies, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Madrid, Spain) and 0.2% antibiotic (Gentamicin/Amphotericin B; Life Technologies). QGP-1cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland), supplemented with 10% FBS, 1% glutamine and 0.2% antibiotic. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 85%. Both cell lines were cultured in 75cm^2 flasks at 37°C in a 5% CO_2 incubator. All experimental procedures in both cell lines were performed at least three times.

Drugs and reagents

Metformin, phenformin, simvastatin, atorvastatin, lovastatin and rosuvastatin were purchased from Sigma Aldrich. Buformin was purchased from Santa Cruz Biotechnology (Texas, USA). All treatments were dissolved in the respective FBS-free-medium and diluted until obtaining final concentrations prior to use (metformin: 10^{-2}M; phenformin and buformin: 5 x10^{-3}M; statins: 10^{-5}M). IGF1 and paclitaxel were purchased from Sigma Aldrich (Madrid, Spain). Drug doses were selected based on *in vitro* dose–response curves (unpublished results) or in previous studies (40, 41).

Cell viability assay

Cells were plated in 100 µL of medium in 96-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later, serum free medium was added during 24 h. After this, biguanides and statins were added into wells in medium with 5% serum. Cell viability was measured using the alamar-blue assay at basal, 24 h, 48 h and 72 h of incubation by measuring the fluorescent signal exciting at 560 nm and reading at 590 nm (Flex Station 3; Molecular Devices). The day of each measurement, cells were incubated for 3 h in 10% alamar blue/serum free-media and then, alamar reduction was measured. After each measurement, medium was replaced immediately by fresh medium. In all cases, cells were seeded per quadruplicate and all assays were repeated a minimum of four times. IGF1 and paclitaxel treatment were used as positive and negative controls, respectively.

Migration capacity assay

The ability BON-1 cells to migrate after 24 h of treatment with biguanides and statins was evaluated by wound healing technique. Briefly, cells were plated at sub-confluence in 12 well plates. Confluent cells were serum-starved for 24 h and after synchronization the wound was made using a 100 µl sterile pipette tip. Cells were incubated for 24 h in FBS free medium. Wound healing was calculated as the area of a rectangle centered in the picture 24 h after the wound vs. the area of the rectangle just after doing the wound, as previously reported (42). At least three experiments per cell line were performed in independent days, in which three random pictures along the wound were acquired per well.

RNA isolation and reverse-transcription

Total RNA from formalin fixed paraffin-embedded (FFPE) samples was isolated using the RNA easy FFPE Kit (Qiagen, Limburg, The Netherlands) according to the manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported (43, 44).

Quantitative real time PCR (qPCR)

cDNA was amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Specifically, expression levels (absolute mRNA copy number/50ng of sample) of insulin receptor (*INSR*), *GLUT4* genes were measured in the cells using previously validated primers (41), and the expression level of each transcript was adjusted by the expression of Beta Actin (*BACT*; used as housekeeping gene). Experiments were performed at least three times.

In human tumor samples, somatostatin system [i.e. somatostatin (*SST*), cortistatin (*CORT*), their receptors *SSTR1*, *SSTR2*, *SSTR3*, *SSTR4*, *SSTR5*, sst5TMD4], and ghrelin system [i.e. ghrelin (*GHRL*), In1-ghrelin variant, ghrelin-o-acyltransferase enzyme (*GOAT*), and the receptors *GHSR1a*/GHSR1b] were evaluated using previously validated primers (41, 45-48). mRNA levels were normalized by 18S in GEP-NETs and by BACT in LCs, as previously described (45, 46). In NET cell lines, the expression of insulin receptor (*INSR*) and glucose transporter GLUT4 was analyzed in response to biguanides and statins treatment using previously validated primers (49, 50).

All samples were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as a negative control. Thermal profile consisted of an initial step at 95°C for 30 s, followed by 50 cycles of denaturation (95°C for 20s) and annealing/elongation (60°C for 20s), and finally, a dissociation cycle (melting curve; 55°C to 95°C, increasing 0.5°C/30 s) to verify that only one product was amplified.

Serotonin assay

BON-1 and QGP-1 cells were cultured in 12-well plates. At 70% confluence, cells were serum starved and after a 24 h incubation period with specific treatments or with vehicle, media were collected and stored at −20°C until measurements. Secretion of serotonin was detected using a serotonin ELISA kit (ALPCO, Salem, NH, USA) following the instructions of the manufacturer.

Apoptosis assay

BON-1 and QGP-1 cells were cultured in 24 well plates. At 70% confluence, cells were serum starved and after 48 h incubation with specific treatments or with vehicle-treated controls in 5% FBS medium, apoptosis levels were measured using a cell death detection ELISA kit (Sigma Aldrich) following the manufacturer's instructions.

Measurement of ERK1/2 and AKT signaling pathways by western blotting

500,000 cells (BON-1 and QGP-1) were cultured in 6-well plates and incubated for 8 min with specific treatments and vehicle-treated controls. Briefly, after the corresponding treatment, medium was removed and cells were washed twice using PBS, detached using a scrapper and immediately lysed in pre-warmed SDS-DTT sample buffer at 65°C (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM DTT and 0.005% bromophenol blue) followed by sonication for 10 s and boiling for 5 min at 95 °C, as previously described (51, 52). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Darmstadt, Germany), and then membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween 20 and incubated with the primary antibodies for total-ERK1/2, p-ERK1/2, total-AKT, p-AKT (Santa Cruz, CA, USA) and then, with the appropriate secondary antibodies [anti-rabbit antibody from Cell Signaling (Danvers, MA, USA)]. Protein analyses were developed using an enhanced chemiluminescence detection system (GE Healthcare, UK) with dyed molecular weight-markers. A densitometric analysis of the bands was carried out with the ImageJ software (53). Relative phosphorylation of ERK and AKT was obtained from normalization of p-ERK1/2 or p-AKT against the total ERK1/2 or AKT, respectively.

Statistical analysis

In functional experiments, results are expressed as percentage vs. vehicle-control (non-treated cells). mRNA levels are expressed as mean ± SEM. Cell survival rate compared to control was assessed by multiple comparison tests. U-Mann Whitney tests were used to evaluate clinical relations within LCs and GEP-NETs samples. Chi-squared test was used to compare categorical data. All statistical analyses were performed using SPSS statistical software version 20 and Graph Pad Prism version 6. p values smaller than 0.05 were considered statistically significant.

RESULTS

Clinical evolution in patients with LCs and GEP-NETs and correlations with T2DM

In the LC group, weight loss was more frequently observed in patients with T2DM than in non-diabetic patients (36.4% vs. 8.6%; p<0.05; Table 1). Likewise, pleura invasion was also higher in T2DM patients (37.5% vs. 2.2%; p<0.05; Table 1). Despite the increased incidence of weight loss in diabetic patients with LC, none of the metformin-treated T2DM patients exhibited this symptom (p<0.05; Table 2). In this cohort, the clinical outcome did not differ in those patients receiving metformin or other antidiabetic treatment (Table 2). Mortality tended to be increased in patients with T2DM (p=0.09; Table 1).

In the GEP-NETs group, an increased incidence of a second neoplasm was observed in the non-diabetic group (25.7% vs. 0%; p<0.05; Table 3). Tumor diameter tended to be higher in patients with T2DM compared to non-diabetic patients (3.4±0.5 vs 2.5±0.2 cm, p=0.06). In addition, the proportion of patients with complete surgical resection was lower in T2DM compared with non-diabetic patients (69.2% vs. 93.8%; p<0.05; Table 3). In this cohort, the clinical outcome of patients treated with metformin was also similar to those treated with other antidiabetic drugs or insulin (Table 4).

None of the rest of clinical parameters evaluated (including functionality or incidental finding), histopathological variables (including, necrosis, local invasion, presence of metastasis, vascular or nerve invasion), tumor grading or evolution parameters (including relapsed disease, disease-free survival and mortality) were associated to the presence of T2DM or the use of metformin in our cohorts of patients with LCs or GEP-NETs.

No clinical, histological or molecular variable was associated to the presence of hyperlipidemia in our cohort of LCs (Table 5) or GEP-NETs (Table 6). A higher proportion of patients treated with statins were free of disease during the follow up (X^2 7.07; p<0.05). None of the other clinical, histological or evolution parameters were associated with the use of statins in our cohorts of patients with LCs or GEP-NETs.

mRNA expression of SST and ghrelin system components in LCs and GEP-NETs and their correlations with T2DM

The mRNA levels of several genes of interest were measured in the tumor tissue obtained from LCs (Figure 1A) and GEP-NET (Figure 1B) patients. In the LCs group, mRNA levels of SST and several receptor subtypes (*SSTR1*, *SSTR2*, *SSTR4, SSTR5* and sst5TMD4), but not of *CORT* or *SSTR3*, were numerically, albeit non-significantly decreased in patients with T2DM compared to the non-diabetic patients (Figure 1A). A similar pattern of expression was observed in the GEP-NETs group, except for the mRNA levels of *SSTR5* (Figure 1B). Interestingly, an overall decreased in the mRNA levels of all SSTRs was found in both, the

Table 1: General characteristics of the LCs patient population

General characteristic		Total (n=81)	Non-diabetic patients (n=61)	T2DM patients (n=14)	*p*
Gender					0.55
	Male	51.8% (42/81)	52.5% (32/61)	50.0% (7/14)	
	Female	48.1% (39/81)	47.5% (29/61)	50.0% (7/14)	
Age (years old)		56.4±15.6	56.1±2.7	58.3±3.7	0.75
Personal history of other tumors		18.7% (14/75)	18.3% (11/60)	21.4% (3/14)	0.52
Smoke habit (Active/ Ex-smoker)		65.5% (38/58)	68.1% (32/47)	60% (6/10)	0.44
Family history of neoplasms		55.6% (5/9)	80.0% (4/6)	20.0% (1/3)	0.40
Weight loss		**14.6% (7/48)**	**8.6% (3/35)**	**36.4% (4/11)**	**0.046**
Functionality		7.5% (4/53)	7.7% (3/39)	8.3% (1/12)	0.67
Incidental		19.2% (10/52)	21.1% (8/38)	16.7% (2/12)	0.55
Maximal tumor diameter (cm)		2.9±2.4	2.8±0.3	3.5±0.8	0.36
Multiple tumors		6.9% (5/72)	5.5% (3/55)	8.3% (1/12)	0.55
Vascular invasion		16.1% (5/50)	17.4% (4/23)	0% (0/6)	0.37
Neural invasion		11.8% (2/17)	8.3% (1/12)	25.0% (1/4)	0.45
Metastasis		25.0% (17/68)	25.0% (13/52)	25.0% (3/12)	0.65
Bronchial infiltration		75.0% (45/60)	90.0% (36/47)	50.0% (4/8)	0.13
Parenchyma infiltration		39.0% (23/59)	37.0% (17/46)	62.5% (5/8)	0.16
Pleura infiltration		**6.8% (4/59)**	**2.2% (1/46)**	**37.5% (3/8)**	**0.008**
Classification					0.17
	Typical	69.4% (34/49)	65.8% (25/38)	77.8% (7/9)	0.69
	Atypical	30.6% (15/49)	34.2% (13/38)	22.2% (2/9)	0.69
Relapsed disease		11.8% (6/51)	12.5% (5/40)	0% (0/9)	0.34
Disease free during follow-up		77.6% (45/58)	77.8% (35/45)	81.8% (9/11)	0.56
Mortality		19.4% (13/67)	15.4% (8/52)	35.7% (5/14)	0.09

p value refers to the comparison between non-diabetic patients and T2DM patients.

Table 2: Metformin in patients with T2DM and LCs

General characteristic	Metformin (n=6)	Other antidiabetic treatment (n=5)	*p*
Weight loss	**0% (0/4)**	**100% (4/4)**	**0.04**
Maximal tumor diameter (cm)	2.6±0.3 cm	6.7±2.7 cm	0.38
Metastasis	0% (0/6)	60.0% (3/5)	0.12
Bronchial infiltration	50.0% (2/4)	50.0% (1/2)	0.8
Parenchyma infiltration	7.05% (3/4)	25.0% (1/4)	0.6
Pleura infiltration	50.0% (2/4)	50.0% (1/2)	0.8
Disease free during follow-up	100% (4/4)	50.0% (2/4)	0.21
Mortality	33.3% (2/6)	60.0% (3/5)	0.39

Table 3: General characteristics of the GEP-NETs patient population

General characteristic		Total (n=100)	Non-diabetic patients (n=70)	T2DM patients (n=17)	*p*
Gender					0.28
	Male	57.0% (57/100)	58.6% (41/70)	47.1% (8/17)	
	Female	43.0% (43/100)	41.4% (29/70)	52.9 % (9/17)	
Age (years)		55.7±17.5	57.8±3.1	55.7±2.2	0.80
Personal history of other tumors		**20.7% (18/87)**	**25.7% (18/70)**	**0% (0/17)**	**0.012**
Smoke habit		67.4% (29/43)	65.7% (23/35)	71.4% (5/7)	0.57
Family history of neoplasms		46.4% (13/28)	45.0% (9/20)	50.0% (4/8)	0.56
Weight loss		38.5% (20/52)	41.5% (17/41)	25.0% (2/8)	0.32
Incidental tumor		40.3% (29/72)	42.3% (22/52)	33.3% (4/12)	0.41
Functionality		31.5% (23/73)	32.1% (17/53)	33.3% (4/12)	0.59
Primary tumor localization					
	Pancreas	36.0% (36/99)	34.3% (24/70)	50.0% (8/16)	
	Stomach	6.0% (6/99)	4.3% (3/70)	6.3% (1/16)	
	Small bowel	19.0% (22/99)	16.3% (14/70)	25.1% (4/16)	
	Colon and rectum	36.0% (36/99)	33.4% (29/70)	0% (0/16)	
Maximal tumor diameter (cm)		2.6±2.2	2.5±0.2	3.4±0.5	0.06
Free surgical border		**87.3% (62/71)**	**93.8% (45/48)**	**69.2% (9/13)**	**0.032**
Multiple tumors		7.5% (4/53)	7.5% (3/40)	0% (0/6)	0.65
Local infiltration		53.1% (43/81)	57.1% (32/56)	42.9% (6/14)	0.25
Vascular invasion		28.4% (21/74)	30.8% (16/52)	25.0% (3/12)	0.50
Neural invasion		29.6% (21/71)	32.7 % (16/49)	16.7% (2/12)	0.24
Metastasis		47.4% (45/95)	45.5% (30/66)	47.1% (8/17)	0.56
Grading (WHO 2010 criteria)					
	Low	46.4% (32/69)	52.2% (24/46)	23.1% (3/13)	0.08
	Intermediate	39.1% (27/69)	34.8% (16/46)	69.2% (9/13)	0.08
	High	14.5% (10/69)	13.0% (6/46)	7.7% (1/13)	0.27
Relapsed disease		34.2% (27/79)	36.8% (21/57)	27.3% (3/11)	0.41
Disease free during follow-up		61.4% (43/70)	60.8% (31/51)	70.0% (7/10)	0.43

p value refers to the comparison between non-diabetic patients and T2DM patients.

LC and GEP-NET groups, but this difference only reached statistical significance in the GEP-NET group (Figure 1C).

Similarly, mRNA levels of all the components of the ghrelin system (*GHRL*, In1-ghrelin, *GOAT* and the receptors *GHSR1a* and GHSR1b) displayed non-significant lower levels in diabetic LCs patients compared with non-diabetic LCs patients (Figure 2A). In GEP-NETs, the mRNA levels of In1-ghrelin, *GOAT* and GHSR1b, but not *GHRL* or *GHSR1a*, also tended to be lower in patients with T2DM (Figure 2B).

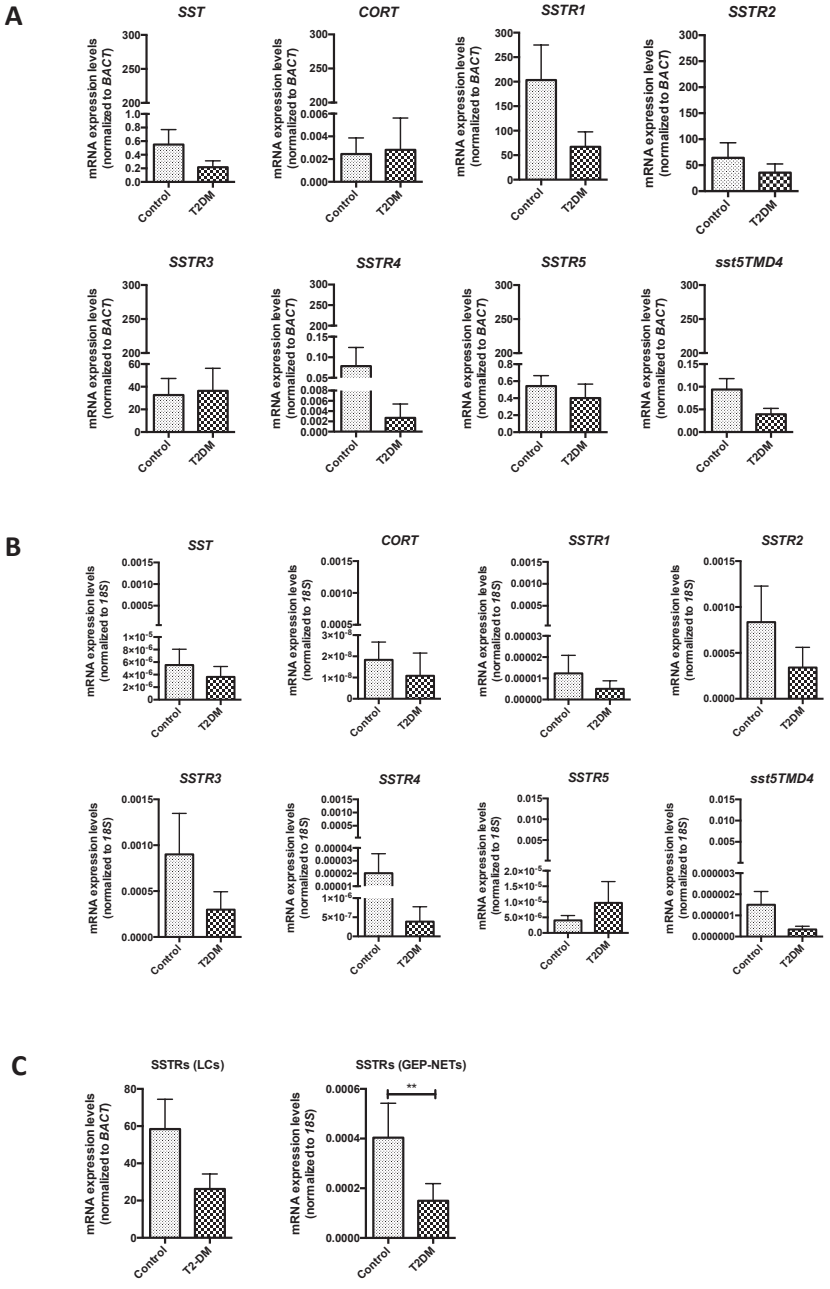


Figure 1: mRNA expression of somatostatin system components in diabetic and non-diabetic patients with LCs (A) and GEP-NETs (B). (C) Total mRNA expression of somatostatin receptors in LCs and GEP-NET patients. The absolute mRNA expression of the different components of the SST system was determined by qPCR in tumor samples (values were normalized to *BACT* in LCs and *18S* in GEP-NETs). Data represent the mean±SEM (**, p<0.01).

Table 4: Metformin in patients with T2DM and GEP-NETs

General characteristic	Metformin (n=9)	Other antidiabetic treatment (n=7)	*p*
Weight loss	25.0% (1/4	33.3% (1/3)	0.71
Maximal tumor diameter (cm)	3.5±0.8 cm	3.5±0.9 cm	0.95
Necrosis	25.0% (1/4)	0% (0/4)	0.50
Vascular invasion	40.0% (2/5)	16.7% (1/6)	0.42
Neural invasion	0% (0/5)	33.3% (2/6)	0. 27
Metastasis	44.4% (4/9)	57.1% (4/7)	0.50
Mortality	55.6% (5/9)	28.6% (2/7)	0.29

Table 5: Statins in patients with LCs

General characteristic	No statins use (n=71)	Statins (n=4)	*p*
Previous other tumor	16.9% (12/71)	50.0% (2/4)	0.16
Weight loss	13.0% (6/46)	50.0% (1/2)	0.27
Maximal tumor diameter (cm)	2.8±0.3	4.6±2.3	0.42
Necrosis	33.3% (8/24)	0% (0/1)	0.68
Metastasis	25.0% (16/64)	25.0% (1/4)	0.74
Bronchial infiltration	74.1% (43/58)	100% (2/2)	0.55
Parenchyma infiltration	40.4% (23/57)	0% (0/2)	0.37
Pleura infiltration	7.0% (4/57)	0% (0/2)	0.87
Disease free during follow-up	78.6% (44/56)	50% (1/2)	0.40
Mortality	17.5% (11/63)	50% (2/4)	0.167

Table 6: Statins in patients with GEP-NETs

General characteristic	No statins use (n=80)	Statins (n=6)	*p*
Weight loss	36.4% (16/44)	60.0% (3/5)	0.29
Maximal tumor diameter (cm)	2.6±0.2 cm	4.3±1.2 cm	0.13
Necrosis	32.0% (8/25)	0% (0/1)	0.69
Peritumoral invasion	52.3% (34/65)	75.0% (3/4)	0.36
Vascular invasion	28.3% (17/60)	33.3% (1/3)	0.64
Neural invasion	28.1% (16/57)	33.3% (1/3)	0.64
Metastasis	44.7%(34/76)	66.7% (4/6)	0.27
Mortality	28% (21/75)	66.7% (4/6)	0.07

Interestingly, a sub-analysis showed that despite the overall expression of SSTRs was significantly lower in the GEP-NETs group with T2DM compared to non-diabetic patients, these levels were not decreased in T2DM patients treated with metformin compared to non-diabetic patients (Figure 3A). Specifically, a non-significant increase in the mRNA levels of *SST*, *CORT*, *SSTR1*, *SSTR2* and *SSTR3* was observed in T2DM patients treated with

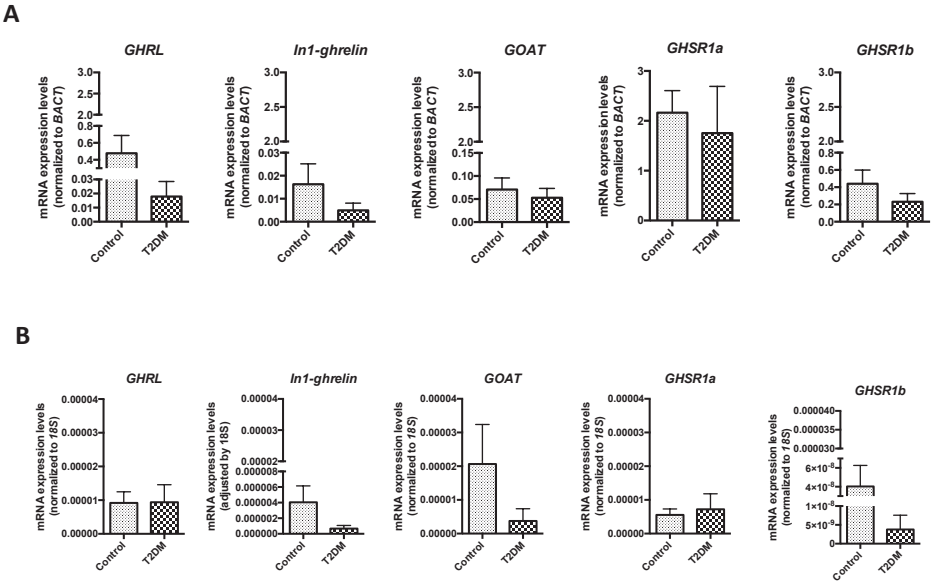


Figure 2: mRNA expression of ghrelin system components in diabetic and non-diabetic patients with LCs (A) and GEP-NETs (B). The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in tumor samples. Data represent the mean±SEM.

metformin compared with T2DM patients without metformin (Figures 3B), as well as in the mRNA levels of *GHRL*, In1-ghrelin and *GHSR1a* (Figures 3C).

Cell survival of PNET cells after treatment with biguanides or statins

All biguanides tested clearly decreased survival rate in both BON-1 and QGP-1 cell lines, in a time-dependent manner (Figure 4A and 4B, respectively). The most remarkable effect was observed in BON-1 cells with phenformin ($5x10^{-3}$ M), which decreased survival rate by 76.6%, 93.1% and 97.13% after 24, 48 and 72h of incubation, respectively. Metformin (10^{-2} M) decreased the survival rate in these cells by 25.1%, 38.1% and 49.4%, whereas buformin ($5x10^{-3}$ M) reduced it by 36.9%, 37.1% and 56.3% after 24, 48 and 72h of incubation, respectively (Figure 4A).

A similar effect was observed in QGP-1 cell line. Specifically, phenformin was also the most effective biguanide since survival rates decreased by 68.2%, 87.4% and 96.9% after 24, 48 and 72h of incubation, respectively; whereas, metformin decreased the survival rate by 24.9%, 45% and 60%, and buformin by 30.7%, 53.0% and 69.7% after 24, 48 and 72 h of incubation, respectively (Figure 4B).

We also analyzed the effect of different statins in cell survival in BON-1 and QGP-1 cell lines (Figure 4C and 4D, respectively). Specifically, a decreased survival rate was observed after 48 and 72h of treatment with simvastatin (10^{-5} M; 21.4% and 34.5%, respectively),

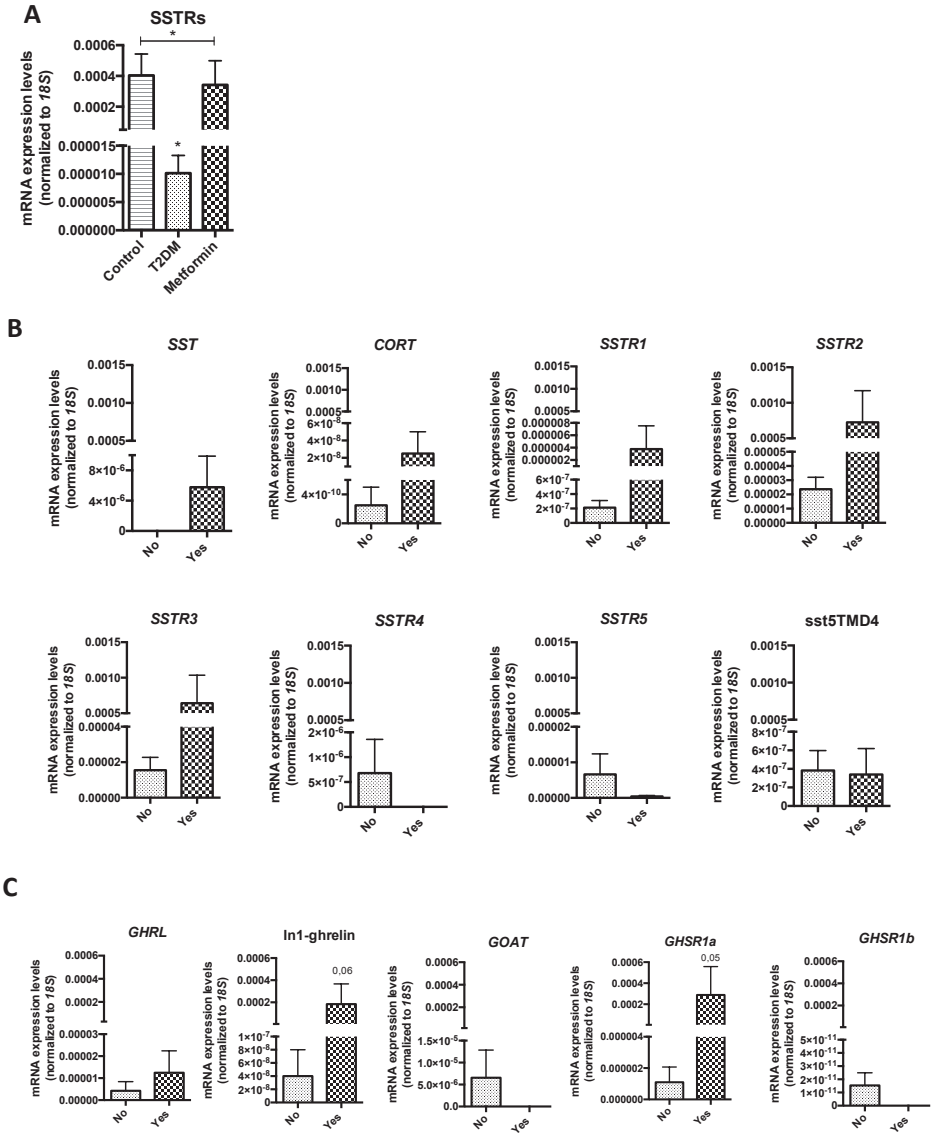


Figure 3: Effects of metformin in patients with T2DM and GEP-NETs. (A) Total mRNA expression of somatostatin receptors in GEP-NETs. Specific SST and ghrelin system components (**B, C** respectively) in GEP-NETs. The absolute mRNA expression of the different components of the SST system was determined by qPCR and normalized to *18S*. mRNA expression was assessed in GEP-NET patients with and without T2DM. Among the last ones, two subgroups were analyzed: those treated with metformin vs those treated with other antidiabetic drugs/insulin. mRNA expression was compared to control. Data represent the mean±SEM (*, $p<0.05$).

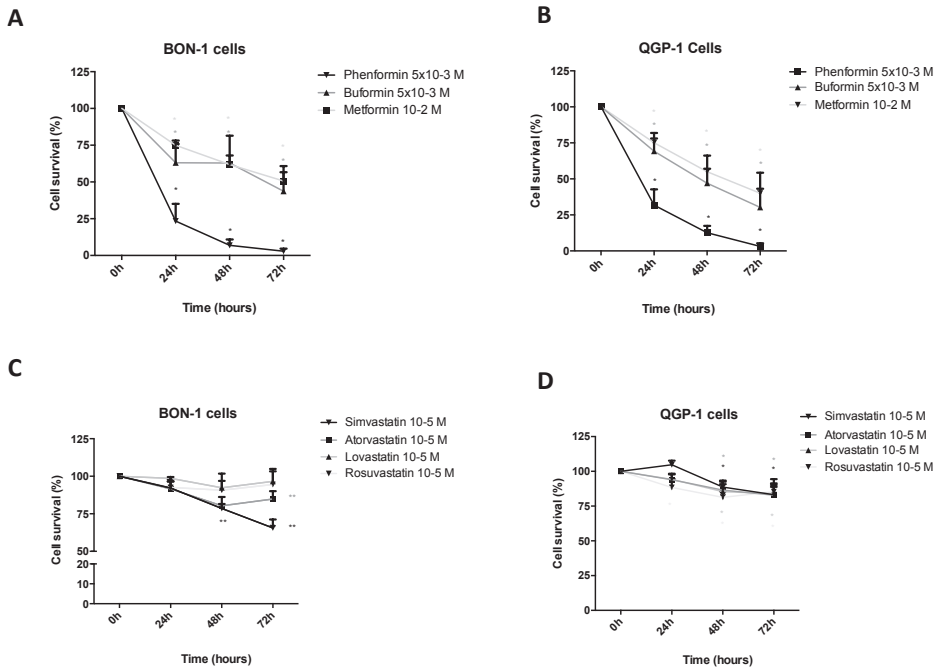


Figure 4: Time-dependent effect on cell viability of biguanides in BON-1 (A) and QGP-1 (B) cell lines; and statins in BON-1 (C) and QGP-1 (D). Cell viability is expressed as cell survival in percentage after 24h, 48 h and 72 h. Cell proliferation rate compared to control was assessed by multiple comparison tests and asterisks (*, p<0.05; **,p<0.01) indicate significant associations.

and after 72h of treatment with atorvastatin (10^{-5} M; 15.2%) in BON-1 cells (Figure 4C), being the effect of simvastatin more pronounced than that of atorvastatin at 72h (p<0.05; Figure 4C). In QGP-1 cells, a reduction in the proliferation rate was observed after 48 and 72h of incubation with all the statins tested (Figure 4D). Thus, simvastatin, atorvastatin, lovastatin and rosuvastatin (10^{-5} M) decreased survival rate ranging from 14.7% to 17.2% after 72h.

Based on their antiproliferative effects, phenformin and simvastatin were chosen as representative compounds of these two classes of drugs in order to perform further functional experiments (i.e. migration, apoptosis and serotonin secretion). Moreover, metformin was also included in these analyses due to its relevance in the clinical practice.

Migration capacity in PNET cells in response to metformin, phenformin and simvastatin treatment

Metformin and simvastatin (24h of incubation) significantly decreased the migration capacity of BON-1 cells (100% and 38.6%, respectively; representative images are depicted in Figure 5A). In contrast, it was not possible to measure the migration capacity in response to

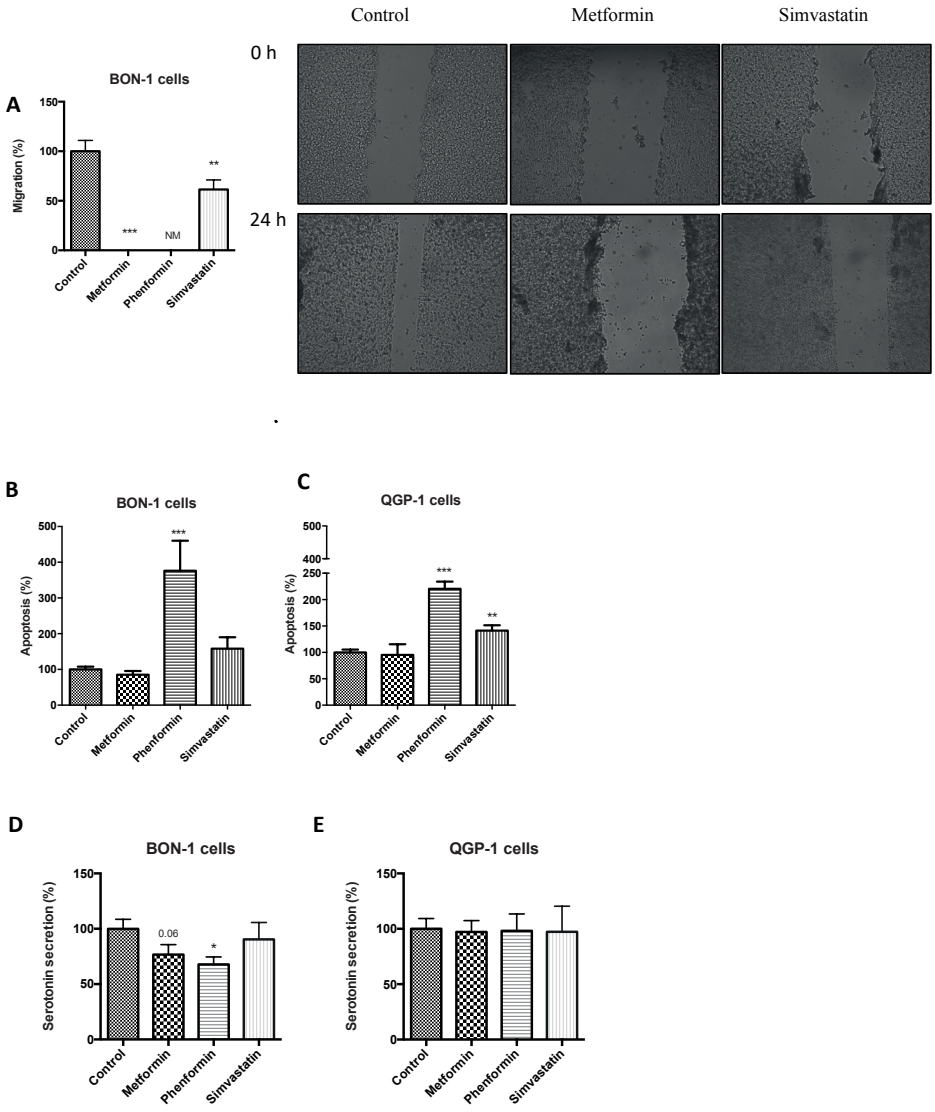


Figure 5: Effect of biguanides and statins on (A) cell migration in BON-1 cells; (B) apoptosis rate in BON-1 (B) and QGP-1 (C) cells; serotonin secretion in BON-1 (D) and QGP-1 (E) cell lines. Migration and serotonin secretion were assessed after 24 hours of incubation; apoptosis rate was evaluated after 48 hours. Representative images of wound healing after 24 hours of treatment are presented in Figure 5A, lower panels. Treatment rates were compared to control by multiple comparison tests and asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate significant associations. Legend NM: non-measurable.

phenformin in BON-1 cells, perhaps due to a treatment-related toxicity of this compound (see below). As previously reported (54) it was not feasible measuring this functional assay on QGP-1 cells since these cells form aggregates/clusters in culture, which do not allow to correctly measure the migration capacity under basal conditions or in response to any given treatment.

The effect of metformin, phenformin and simvastatin treatment on apoptosis

In BON-1 cells, phenformin caused a three-fold increase in apoptosis (Figure 5B). However, metformin or simvastatin treatment did not alter apoptosis in BON-1 cells. In QGP-1 cells, a two-fold increase in apoptosis was also observed in response to phenformin (Figure 5C). In addition, simvastatin increased apoptotic rate in QGP-1 cells by 58.1% (Figure 5C). Conversely, metformin treatment did not alter apoptosis in QGP-1 cells.

Effect of biguanides and statins on serotonin secretion in PNET cell lines

In BON-1 cells, phenformin, but not simvastatin decreased serotonin secretion after 24h of incubation ($p<0.05$; Figure 5D). Metformin treatment also tended to decrease serotonin release ($p=0.06$) (Figure 5D). In contrast, none of these treatments altered serotonin secretion from QGP-1 cells (Figure 5E).

Effects of metformin, phenformin and simvastatin on ERK1/2 and AKT signaling pathways

To start exploring the signaling pathways affected by biguanides (metformin and phenformin) and simvastatin to induce their functional actions in NET cells, the levels of phosphorylation of AKT and ERK were evaluated. In BON-1 cells, both biguanides and simvastatin similarly decreased phosphorylation levels of AKT and ERK compared to controls (Figure 6A). In marked contrast, in QGP-1 cells, only phenformin and simvastatin decreased phosphorylation levels of ERK without altering those of AKT (Figure 6B).

The effect of metformin, phenformin and simvastatin in the expression of key genes in PNET pathophysiology

In BON-1 cells, metformin and phenformin severely decreased the mRNA levels of *INSR* ($p<0.001$) (Figure 7A). Also, a trend to an increase in the expression *GLUT-4* was observed in response to phenformin (Figure 6A). In QGP-1 cells, *GLUT-4* expression was increased in response to both biguanides and simvastatin, but this difference only reached statistical significance in the case of phenformin (Figure 7B). Whereas, no significant changes were observed in the expression of *INSR* in QGP-1 cells in response to these compounds (Figure 7B). Finally, metformin treatment did not significantly alter the expression of SSTRs in BON-1 and QGP-1 cells (data not shown).

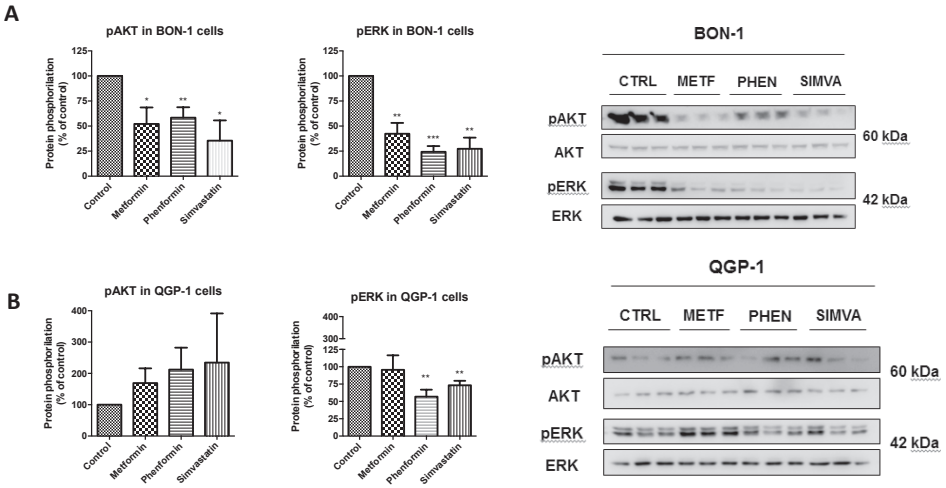


Figure 6: Effects of biguanides and statins on (A) AKT and ERK phosphorylation in BON-1 (A) and QGP-1 (B) cells. Phosphorylation levels compared to control was assessed by multiple comparison tests and asterisks (*, p<0.05; **, p<0.01; ***, p<0.001) indicate significant associations.

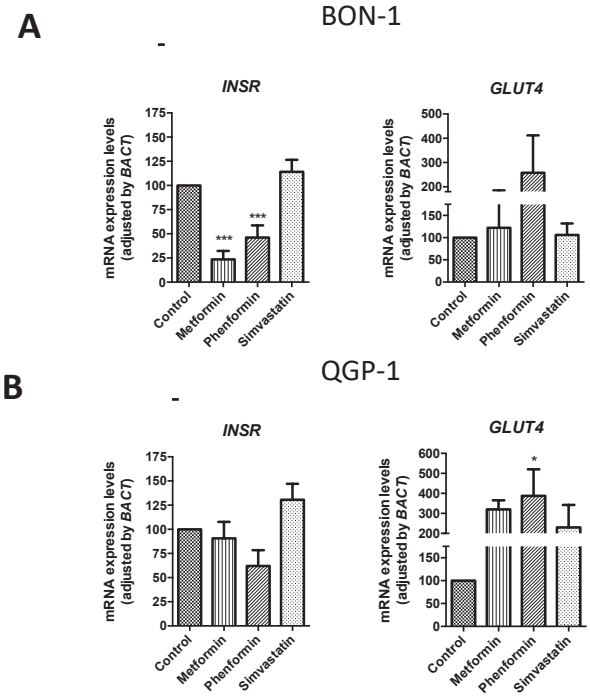


Figure 7: Effects of biguanides and statins on mRNA expression in (A) BON-1 and (B) QGP-1 cells. mRNA expression compared to control was assessed by multiple comparison tests and asterisks (*, p<0.05; **, ***, p<0.001) indicate significant associations.

DISCUSSION

NETs are a widely heterogeneous group of neoplasms, which are frequently diagnosed at an advanced stage of disease (55). Therapeutic options for advanced metastasized NETs include somatostatin analogs, interferon-α, chemotherapy and peptide receptor therapy (55, 56). In the last years, target-directed therapies have increased the therapeutic spectrum for progressive NETs (e.g. sunitinib as tyrosine kinase inhibitor and everolimus for the mTOR pathway inhibition) (57-59). However, despite the improvements in progression-free survival of these therapeutic options, their effect on the overall survival is still controversial (60). Therefore, novel treatments are still required, especially for patients with advanced disease.

The incidence of metabolic syndrome is continuously increasing, reaching almost 35% in some countries, with a consequent increase in the prevalence of some types of cancer (61). T2DM has been also related to an increased risk of malignancies (62, 63) and is frequently developed in patients using everolimus and some somatostatin analogs (64, 65). In this context, metformin is one of the most widely prescribed oral hypoglycemic agents and, in the last years, it has received an increased attention because of its potential antitumorigenic effects (5, 66). Likewise, some publications have described that statins exert an inhibitory effect on tumor-induced angiogenesis and an antitumor effect in cellular/animal models of human cancer (22, 67); however, other studies have also suggested a potential risk of cancer when statins are used (68). In this sense, the present study, although using a limited number of samples, is the first that: 1) assesses the association of T2DM with clinical evolution parameters in patients with different NET types (LCs and GEP-NETs); 2) evaluates the expression levels of all the components of two key regulatory systems (SST and ghrelin) in the tumor tissue of patients with these two NET types, in relation to T2DM and metformin treatment; and 3) analyzes and compares the effects of different biguanides and statins in key functional parameters in two representative models of NET cell lines, BON-1 and QGP-1 cells.

T2DM is linked to relevant defects in the INSR signaling pathway, which regulates growth and metabolic responses in insulin target cells and tissues (69). Some epidemiological studies have described an increased risk for several types of cancer (breast, colon, rectum, liver, and pancreas) in insulin-resistant patients (70). Remarkably, in our cohort of patients, a more aggressive pattern in LCs (increased incidence of pleura invasion) and increased tumor size in GEP-NETs were observed in patients with T2DM when compared to non-diabetic patients, suggesting a possible association between T2DM and NET pathophysiology.

Since insulin is related to increased risk of cancer, treatment options targeting this pathway could be effective in cancer prevention (71). Indeed, a meta-analysis showed a 31% reduction in overall cancer incidence and a 34% decline in cancer mortality in patients with diabetes treated with metformin (72). A retrospective study in patients with T2DM and NETs showed lower recurrence rate in those treated with metformin compared with non-metformin treated or non-diabetic patients (72). Moreover, in a cohort of pancre-

atic NET patients receiving everolimus and octreotide LAR, progression free survival was higher in patients treated with metformin compared to other drugs (73). To the best of our knowledge, no other specific reports in NETs have been published yet. In our cohort, metformin appeared to avoid weight loss in patients with LCs and T2DM. Interestingly, the numerical records assessed in patients with LCs receiving metformin suggested that tumor size were smaller, incidence of metastasis was lower and disease-free during follow-up was higher than in non-metformin treated patients; however, these results did not reach statistical significance, likely due to the limited size of the groups. In contrast, no association was observed between clinical/histological variables and the use of metformin in the GEP-NETs group. Obviously, we should underline that the main limitation of this work might be the limited number of patients with T2DM and those treated with metformin included in the analysis, although the size of the total cohort evaluated was large enough for making general comparisons. Therefore, like other studies reporting a limited cohort of samples (73), the results of this study should be interpreted with caution.

Novel mechanisms of action have been proposed for metformin in recent years. Among them, the induction of the expression of the glucagon-like peptide 1 (GLP-1) receptor on pancreas β-cells was described (74). To the best of our knowledge, this is the first report showing that the expression of several SSTR subtypes is reduced in NETs from diabetic patients (LCs and GEP-NETs) compared with those from non-diabetic patients, and most importantly, that the overall expression of SSTR is significantly increased in LCs from diabetic patients treated with metformin compared with LCs from diabetic patients without metformin treatment. In fact, these expression levels of SSTR in LCs from diabetic patients treated with metformin achieved the levels observed in LCs from patients without T2DM. These novel results provide suggestive evidence that metformin treatment could increase SSTRs expression in NETs in diabetic patients, which might be important from a clinical point of view, in that a previous study has suggested that metformin could have potential synergistic effect when combined with somatostatin analogs via the inhibition of PI3K/AKT/mTOR axis (73). Thus, it will be worth to elucidate in the future the mechanisms involved in the capacity of metformin to regulate the expression of SSTRs, as well as a putative synergistic effect between somatostatin analogs and metformin. In this sense, we analyzed the SSTR expression in BON1 and QGP1 cells after metformin treatment, but we did not observe changes in SSTRs mRNA expression levels, which could be in line with the idea that metformin could reverse the changes previously altered under diabetic conditions and maybe only have reduced potential to modulate basal expression of SSTRs. In addition, it has to be noted that pre- or co-treatment with biguanides and statins was not evaluated in this study, since the anti-proliferative *in vitro* response to somatostatin analogs is limited in these NET cell lines (45, 96, 97).

Biguanides increase insulin sensitivity as well as glucose use by peripheral tissues (3). Antitumoral effects of metformin and phenformin have been evaluated in *in vitro* and *in vivo*

studies, and metformin is also being tested as an adjuvant therapy to classic chemotherapeutic regimens (66, 75). Specifically, an earlier study showed that metformin inhibited cell proliferation in pancreatic, bronchopulmonary and midgut neuroendocrine tumor cell lines in a dose-dependent manner, wherein this antitumoral effects appeared to be mediated via inhibition of mTORC1 signaling (12). Metformin has been also shown to inhibit breast cancer cell growth *in vitro* in an AMPK-dependent manner, in association with a decreased mTOR activation (76). In our study, we also observed a time-dependent antiproliferative effect of different biguanides in PNET cell lines. Similarly, our study revealed that biguanides were able to exert additional, beneficial effects on NET cell function by measuring other relevant functional endpoints (i.e. migration capacity and apoptotic rate). These results support and extend previous data showing that metformin exerted antitumoral actions *in vitro* by modulating cell proliferation and apoptosis in breast cancer cells (77). However, we found that phenformin, but not metformin, was able to increase apoptosis in both NET cell lines, which is partially in agreement with previous data indicating that apoptosis induced by metformin would differ depending on the NET cell type (12). We also found that metformin and phenformin were able to decrease serotonin secretion in BON-1, but not in QGP-1 cells. Although the exact mechanisms are still to be elucidated, these results could be clinically relevant in patients with carcinoid syndrome, since elevated serotonin levels are directly associated with symptoms in this pathology (78). In this sense, we should remark that this is not the first time that different results are observed in the functional response of BON-1 and QGP-1 cells (45, 79-81), which further emphasizes their potential distinct value to study the intrinsic heterogeneity of NETs. Indeed, the reason for these differences is still unknown, but could be related to the distinct expression pattern of key regulatory systems (e.g. SST, ghrelin, IGF-I, etc.) (79-82) and/or to the different activation or signaling of these NET cells in response to the same treatment as it has been previously observed, for instance, for SST analogs (i.e. octreotide and pasireotide) (79, 80).

Statins can also exert antitumoral actions. Thus, a phase II trial has reported a statin-induced anti-proliferative effect in breast cancer (83). As well, the antiproliferative effect of statins has also been reported in several cancer cell lines including cervical (84), leukemic natural killer (85), cholangiocarcinoma (86) and prostate (87). In line with these previous studies, we observed here, for the first time, that different statins exerted a clear antiproliferative effect in NET cells. Additionally, we found that simvastatin was able to significantly increased apoptosis levels in QGP-1 cells, an effect that has been also previously described in cervical cancer, leukemia, natural killer and cholangiocarcinoma cell lines (83-85).

It is well known that the PI3K/AKT/mTORC1 pathway exerts important roles in NETs pathogenesis (88). In LCs, metformin inhibited AKT, ERK and mTOR pathways, suggesting that its antiproliferative effects can be both AMPK-dependent and AMPK-independent (89). In fact, Vlotides et al. have suggested that the functional effect of metformin is cell type-dependent, since they reported that AMPK and AKT phosphorylation was elevated in

pancreatic and midgut NET-cell lines in response to metformin (48h of incubation) but this effect was not observed in bronchopulmonary neuroendocrine cells (12). Interestingly, it was also suggested that the inhibition of the mTOR pathway was associated to the induction of GSK3 phosphorylation following the ERK or AKT pathway (12). In our study, we observed an inhibition of phosphorylated AKT and ERK pathways after treating cells with biguanides (and also with simvastatin), which also reveals the AMPK-dependent and -independent effects of these drugs in NET cells. It should be mentioned that the differences between our results and those reported by Vlotides et al. may be related to the drug-incubation period (8min vs. 48h). However, as found in our study, cell inhibition of the ERK pathway has been also reported in non-small lung cancer and cholangiocarcinoma cell lines with concomitant induction of apoptosis (86, 90).

Nowadays, the mechanisms linking T2DM and cancer comprise a most exciting and interesting research topic. It has been proposed that chronic hyperinsulinemia may promote the development of neoplasms via abnormal stimulation of multiple cellular signaling cascades by insulin, enhancing growth factor-dependent cell proliferation and/or modifying cell metabolism (66). In our results, we observed changes in the molecular expression of key genes involved in tumor aggressiveness (e.g. *INSR* and/or *GLUT-4*) in response to metformin or phenformin, but not simvastatin, suggesting a putative modulatory effect of biguanides in these signaling pathways. In line with this, some studies have suggested that the anti-proliferative effect of statins in cancer cell lines might be associated to a cell-cycle regulatory effects (83), epigenetic alterations (91), or to gene expression modifications of cancer signaling (92). However, the effects of simvastatin treatment on these regulatory endpoints, as well as whether metformin and simvastatin could play synergistic effects in NETs (which has been demonstrated in different tumor pathologies (93-95)) could not be evaluated in our study, but deserve further attention in the future.

In sum, our study using a limited cohort of patients reveals a potential association between key clinical parameters of NET aggressiveness (i.e. incidence of pleura invasion or metastasis, tumor size, etc.) and the presence of diabetes and/or treatment with antidiabetic drugs in patients with different NET types (LCs and GEP-NETs). Moreover, this study provides evidence that the expression of multiple components of two key regulatory systems for the pathophysiology of NETs, the SST and ghrelin systems, are modulated in diabetic patients with LCs and GEP-NETs compared to non-diabetic patients. Finally, our results also showed that different biguanides and statins are capable to directly exert clear antitumoral actions in NET cells, probably due to their effect on cell survival, cell migration, apoptosis, gene expression and metabolic pathways modifications. Therefore, since metformin and statins are low-cost commercially available drugs, with a safe profile and large experience in their clinical use, our present results invite to further explore their potential value as adjuvant therapy for the treatment of NET patients.

REFERENCES

1. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol. 2018;14(2):88-98.
2. Braun S, Bitton-Worms K, LeRoith D. The link between the metabolic syndrome and cancer. Int J Biol Sci. 2011;7(7):1003-15.
3. Song R. Mechanism of Metformin: A Tale of Two Sites. Diabetes Care. 2016;39(2):187-9.
4. Bailey CJ. Biguanides and NIDDM. Diabetes Care. 1992;15(6):755-72.
5. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. BMJ. 2005;330(7503):1304-5.
6. Li D, Yeung SC, Hassan MM, Konopleva M, Abbruzzese JL. Antidiabetic therapies affect risk of pancreatic cancer. Gastroenterology. 2009;137(2):482-8.
7. Lee MS, Hsu CC, Wahlqvist ML, Tsai HN, Chang YH, Huang YC. Type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in Taiwanese: a representative population prospective cohort study of 800,000 individuals. BMC Cancer. 2011;11:20.
8. Mazzone PJ, Rai H, Beukemann M, Xu M, Jain A, Sasidhar M. The effect of metformin and thiazolidinedione use on lung cancer in diabetics. BMC Cancer. 2012;12:410.
9. Col NF, Ochs L, Springmann V, Aragaki AK, Chlebowski RT. Metformin and breast cancer risk: a meta-analysis and critical literature review. Breast Cancer Res Treat. 2012;135(3): 639-46.
10. Ma S, Zheng Y, Xiao Y, Zhou P, Tan H. Meta-analysis of studies using metformin as a reducer for liver cancer risk in diabetic patients. Medicine (Baltimore). 2017;96(19):e6888.
11. Li X, Li T, Liu Z, Gou S, Wang C. The effect of metformin on survival of patients with pancreatic cancer: a meta-analysis. Sci Rep. 2017;7(1):5825.
12. Vlotides G, Tanyeri A, Spampatti M, Zitzmann K, Chourdakis M, Sptt C, et al. Anticancer effects of metformin on neuroendocrine tumor cells in vitro. Hormones (Athens). 2014;13(4): 498-508.
13. Soares HP, Ni Y, Kisfalvi K, Sinnett-Smith J, Rozengurt E. Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells. PLoS One. 2013;8(2):e57289.
14. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108(8):1167-74.
15. Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, et al. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. Cell Metab. 2010;11(6):554-65.
16. Micic D, Cvijovic G, Trajkovic V, Duntas LH, Polovina S. Metformin: its emerging role in oncology. Hormones (Athens). 2011;10(1):5-15.
17. Jalving M, Gietema JA, Lefrandt JD, de Jong S, Reyners AK, Gans RO, et al. Metformin: taking away the candy for cancer? Eur J Cancer. 2010;46(13):2369-80.
18. Emami Riedmaier A, Fisel P, Nies AT, Schaeffeler E, Schwab M. Metformin and cancer: from the old medicine cabinet to pharmacological pitfalls and prospects. Trends Pharmacol Sci. 2013;34(2):126-35.
19. Dowling RJ, Niraula S, Stambolic V, Goodwin PJ. Metformin in cancer: translational challenges. J Mol Endocrinol. 2012;48(3):R31-43.

20. Bost F, Sahra IB, Le Marchand-Brustel Y, Tanti JF. Metformin and cancer therapy. Curr Opin Oncol. 2012;24(1):103-8.
21. Luengo A, Sullivan LB, Heiden MG. Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy. BMC Biol. 2014;12:82.
22. Gazzerro P, Proto MC, Gangemi G, Malfitano AM, Ciaglia E, Pisanti S, et al. Pharmacological actions of statins: a critical appraisal in the management of cancer. Pharmacol Rev. 2012;64(1): 102-46.
23. Mach F. Toward a role for statins in immunomodulation. Mol Interv. 2002;2(8):478-80.
24. Farmer JA. Pleiotropic effects of statins. Curr Atheroscler Rep. 2000;2(3):208-17.
25. Bellosta S, Ferri N, Bernini F, Paoletti R, Corsini A. Non-lipid-related effects of statins. Ann Med. 2000;32(3):164-76.
26. Hess DC, Fagan SC. Pharmacology and clinical experience with simvastatin. Expert Opin Pharmacother. 2001;2(1):153-63.
27. Brozovic A, Fritz G, Christmann M, Zisowsky J, Jaehde U, Osmak M, et al. Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance. Int J Cancer. 2004;112(6):974-85.
28. Koyuturk M, Ersoz M, Altiok N. Simvastatin induces apoptosis in human breast cancer cells: p53 and estrogen receptor independent pathway requiring signalling through JNK. Cancer Lett. 2007;250(2):220-8.
29. Denoyelle C, Vasse M, Korner M, Mishal Z, Ganne F, Vannier JP, et al. Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study. Carcinogenesis. 2001;22(8):1139-48.
30. Bjarnadottir O, Romero Q, Bendahl PO, Jirstrom K, Ryden L, Loman N, et al. Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial. Breast Cancer Res Treat. 2013;138(2):499-508.
31. Garwood ER, Kumar AS, Baehner FL, Moore DH, Au A, Hylton N, et al. Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer. Breast Cancer Res Treat. 2010;119(1):137-44.
32. Maj M, Czajkowski R, Zegarska B, Kowaliszyn B, Pokrywczynska M, Drewa T. Anti-proliferative and cytotoxic activity of rosuvastatin against melanoma cells. Postepy Dermatol Alergol. 2016;33(4):257-62.
33. Jang HJ, Hong EM, Park SW, Byun HW, Koh DH, Choi MH, et al. Statin induces apoptosis of human colon cancer cells and downregulation of insulin-like growth factor 1 receptor via proapoptotic ERK activation. Oncol Lett. 2016;12(1):250-6.
34. Hu HJ, Zhou SH, Liu QM. Treatment of pheochromocytoma blockade of MAPK pathway inhibition in the NF-kappaB pathway and bFGF - effect of statins on pheochromocytoma patients. Int J Cardiol. 2015;182:161-2.
35. Fliedner SM, Engel T, Lendvai NK, Shankavaram U, Nolting S, Wesley R, et al. Anti-cancer potential of MAPK pathway inhibition in paragangliomas-effect of different statins on mouse pheochromocytoma cells. PLoS One. 2014;9(5):e97712.
36. Kaku M, Nishiyama T, Yagawa K, Abe M. Establishment of a carcinoembryonic antigen-producing cell line from human pancreatic carcinoma. Gan. 1980;71(5):596-601.
37. Iguchi H, Hayashi I, Kono A. A somatostatin-secreting cell line established from a human pancreatic islet cell carcinoma (somatostatinoma): release experiment and immunohistochemical study. Cancer Res. 1990;50(12):3691-3.

38. Evers BM, Townsend CM, Jr., Upp JR, Allen E, Hurlbut SC, Kim SW, et al. Establishment and characterization of a human carcinoid in nude mice and effect of various agents on tumor growth. Gastroenterology. 1991;101(2):303-11.
39. Hofving T, Arvidsson Y, Almobarak B, Inge L, Pfragner R, Persson M, et al. The neuroendocrine phenotype, genomic profile and therapeutic sensitivity of GEPNET cell lines. Endocr Relat Cancer. 2018;25(3):367-80.
40. Hormaechea-Agulla D, Gahete MD, Jimenez-Vacas JM, Gomez-Gomez E, Ibanez-Costa A, F LL, et al. The oncogenic role of the In1-ghrelin splicing variant in prostate cancer aggressiveness. Mol Cancer. 2017;16(1):146.
41. Ibanez-Costa A, Gahete MD, Rivero-Cortes E, Rincon-Fernandez D, Nelson R, Beltran M, et al. In1-ghrelin splicing variant is overexpressed in pituitary adenomas and increases their aggressive features. Scientific reports. 2015;5:8714.
42. Sarmento-Cabral A, F LL, Gahete MD, Castano JP, Luque RM. Metformin Reduces Prostate Tumor Growth, in a Diet-Dependent Manner, by Modulating Multiple Signaling Pathways. Mol Cancer Res. 2017;15(7):862-74.
43. Duran-Prado M, Gahete MD, Martinez-Fuentes AJ, Luque RM, Quintero A, Webb SM, et al. Identification and characterization of two novel truncated but functional isoforms of the somatostatin receptor subtype 5 differentially present in pituitary tumors. J Clin Endocrinol Metab. 2009;94(7):2634-43.
44. Martinez-Fuentes AJ, Moreno-Fernandez J, Vazquez-Martinez R, Duran-Prado M, de la Riva A, Tena-Sempere M, et al. Ghrelin is produced by and directly activates corticotrope cells from adrenocorticotropin-secreting adenomas. J Clin Endocrinol Metab. 2006;91(6):2225-31.
45. Herrera-Martinez AD, Gahete MD, Pedraza-Arevalo S, Sanchez-Sanchez R, Ortega-Salas R, Serrano-Blanch R, et al. Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors. Endocrine. 2018;59(2):426-37.
46. Herrera-Martinez AD, Gahete MD, Sanchez-Sanchez R, Salas RO, Serrano-Blanch R, Salvatierra A, et al. The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features. Lung Cancer. 2017;109:128-36.
47. Hormaechea-Agulla D, Gomez-Gomez E, Ibanez-Costa A, Carrasco-Valiente J, Rivero-Cortes E, F LL, et al. Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer, and its levels are associated with patient's metabolic status: Potential value as a non-invasive biomarker. Cancer Lett. 2016;383(1):125-34.
48. Sampedro-Nunez M, Luque RM, Ramos-Levi AM, Gahete MD, Serrano-Somavilla A, Villa-Osaba A, et al. Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors. Oncotarget. 2016;7(6):6593-608.
49. Gahete MD, Cordoba-Chacon J, Lin Q, Bruning JC, Kahn CR, Castano JP, et al. Insulin and IGF-I inhibit GH synthesis and release in vitro and in vivo by separate mechanisms. Endocrinology. 2013;154(7):2410-20.
50. F LL, Sarmento-Cabral A, Herrero-Aguayo V, Gahete MD, Castano JP, Luque RM. Obesity and metabolic dysfunction severely influence prostate cell function: role of insulin and IGF1. J Cell Mol Med. 2017;21(9):1893-904.
51. Gahete MD, Cordoba-Chacon J, Anadumaka CV, Lin Q, Bruning JC, Kahn CR, et al. Elevated GH/IGF-I, due to somatotrope-specific loss of both IGF-I and insulin receptors, alters glucose homeostasis and insulin sensitivity in a diet-dependent manner. Endocrinology. 2011; 152(12):4825-37.

52. Duran-Prado M, Gahete MD, Hergueta-Redondo M, Martinez-Fuentes AJ, Cordoba-Chacon J, Palacios J, et al. The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells. Oncogene. 2012; 31(16):2049-61.
53. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671-5.
54. Kim JT, Li J, Song J, Lee EY, Weiss HL, Townsend CM, Jr., et al. Differential expression and tumorigenic function of neurotensin receptor 1 in neuroendocrine tumor cells. Oncotarget. 2015;6(29):26960-70.
55. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. Gastroenterology. 2008;135(5):1469-92.
56. Kam BL, Teunissen JJ, Krenning EP, de Herder WW, Khan S, van Vliet EI, et al. Lutetium-labelled peptides for therapy of neuroendocrine tumours. Eur J Nucl Med Mol Imaging. 2012; 39 Suppl 1:S103-12.
57. Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Van Cutsem E, et al. Everolimus for advanced pancreatic neuroendocrine tumors. N Engl J Med. 2011;364(6):514-23.
58. Raymond E, Dahan L, Raoul JL, Bang YJ, Borbath I, Lombard-Bohas C, et al. Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. N Engl J Med. 2011;364(6): 501-13.
59. Zitzmann K, De Toni EN, Brand S, Goke B, Meinecke J, Spottl G, et al. The novel mTOR inhibitor RAD001 (everolimus) induces antiproliferative effects in human pancreatic neuroendocrine tumor cells. Neuroendocrinology. 2007;85(1):54-60.
60. Valle JW, Eatock M, Clueit B, Gabriel Z, Ferdinand R, Mitchell S. A systematic review of non-surgical treatments for pancreatic neuroendocrine tumours. Cancer Treat Rev. 2014;40(3): 376-89.
61. Aguilar M, Bhuket T, Torres S, Liu B, Wong RJ. Prevalence of the metabolic syndrome in the United States, 2003-2012. Jama. 2015;313(19):1973-4.
62. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. CA Cancer J Clin. 2010;60(4):207-21.
63. Choi JB, Moon HW, Park YH, Bae WJ, Cho HJ, Hong SH, et al. The Impact of Diabetes on the Risk of Prostate Cancer Development according to Body Mass Index: A 10-year Nationwide Cohort Study. J Cancer. 2016;7(14):2061-6.
64. Yao JC, Lombard-Bohas C, Baudin E, Kvols LK, Rougier P, Ruszniewski P, et al. Daily oral everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. J Clin Oncol. 2010;28(1):69-76.
65. Pavel ME, Baudin E, Oberg KE, Hainsworth JD, Voi M, Rouyrre N, et al. Efficacy of everolimus plus octreotide LAR in patients with advanced neuroendocrine tumor and carcinoid syndrome: final overall survival from the randomized, placebo-controlled phase 3 RADIANT-2 study. Ann Oncol. 2017.
66. Kourelis TV, Siegel RD. Metformin and cancer: new applications for an old drug. Med Oncol. 2012;29(2):1314-27.
67. Wang A, Aragaki AK, Tang JY, Kurian AW, Manson JE, Chlebowski RT, et al. Statin use and all-cancer survival: prospective results from the Women's Health Initiative. British journal of cancer. 2016;115(1):129-35.

68. Fujimoto M, Higuchi T, Hosomi K, Takada M. Association between statin use and cancer: data mining of a spontaneous reporting database and a claims database. International journal of medical sciences. 2015;12(3):223-33.
69. Semple RK, Savage DB, Cochran EK, Gorden P, O'Rahilly S. Genetic syndromes of severe insulin resistance. Endocrine reviews. 2011;32(4):498-514.
70. Cowey S, Hardy RW. The metabolic syndrome: A high-risk state for cancer? The American journal of pathology. 2006;169(5):1505-22.
71. Kasznicki J, Sliwinska A, Drzewoski J. Metformin in cancer prevention and therapy. Ann Transl Med. 2014;2(6):57.
72. Gandini S, Puntoni M, Heckman-Stoddard BM, Dunn BK, Ford L, DeCensi A, et al. Metformin and cancer risk and mortality: a systematic review and meta-analysis taking into account biases and confounders. Cancer Prev Res (Phila). 2014;7(9):867-85.
73. Pusceddu S, Buzzoni R, Vernieri C, Concas L, Marceglia S, Giacomelli L, et al. Metformin with everolimus and octreotide in pancreatic neuroendocrine tumor patients with diabetes. Future Oncol. 2016;12(10):1251-60.
74. Hur KY, Lee MS. New mechanisms of metformin action: Focusing on mitochondria and the gut. J Diabetes Investig. 2015;6(6):600-9.
75. Shikhman AR, Lebedev KA, Dil'man VM. [Inhibiting effect of phenformin (phenethyl biguanide) on the growth of Ehrlich carcinoma]. Vopr Onkol. 1981;27(2):67-9.
76. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer research. 2006;66(21):10269-73.
77. Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, et al. Metformin induces unique biological and molecular responses in triple negative breast cancer cells. Cell Cycle. 2009;8(13): 2031-40.
78. O'Toole D, Ducreux M, Bommelaer G, Wemeau JL, Bouche O, Catus F, et al. Treatment of carcinoid syndrome: a prospective crossover evaluation of lanreotide versus octreotide in terms of efficacy, patient acceptability, and tolerance. Cancer. 2000;88(4):770-6.
79. Sampedro-Nunez M, Luque RM, Ramos-Levi AM, Gahete MD, Serrano-Somavilla A, Villa-Osaba A, et al. Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors. Oncotarget. 2015.
80. van Adrichem RC, de Herder WW, Kamp K, Brugts MP, de Krijger RR, Sprij-Mooij DM, et al. Effects of Somatostatin Analogs and Dopamine Agonists on Insulin-Like Growth Factor 2-Induced Insulin Receptor Isoform A Activation by Gastroenteropancreatic Neuroendocrine Tumor Cells. Neuroendocrinology. 2016;103(6):815-25.
81. Luque RM, Sampedro-Nunez M, Gahete MD, Ramos-Levi A, Ibanez-Costa A, Rivero-Cortes E, et al. In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters. Oncotarget. 2015;6(23):19619-33.
82. Fraenkel M, Kim M, Faggiano A, de Herder WW, Valk GD, Knowledge N. Incidence of gastroenteropancreatic neuroendocrine tumours: a systematic review of the literature. Endocr Relat Cancer. 2014;21(3):R153-63.
83. Feldt M, Bjarnadottir O, Kimbung S, Jirstrom K, Bendahl PO, Veerla S, et al. Statin-induced anti-proliferative effects via cyclin D1 and p27 in a window-of-opportunity breast cancer trial. J Transl Med. 2015;13:133.

84. Crescencio ME, Rodriguez E, Paez A, Masso FA, Montano LF, Lopez-Marure R. Statins inhibit the proliferation and induce cell death of human papilloma virus positive and negative cervical cancer cells. Int J Biomed Sci. 2009;5(4):411-20.
85. Crosbie J, Magnussen M, Dornbier R, Iannone A, Steele TA. Statins inhibit proliferation and cytotoxicity of a human leukemic natural killer cell line. Biomark Res. 2013;1(1):33.
86. Kamigaki M, Sasaki T, Serikawa M, Inoue M, Kobayashi K, Itsuki H, et al. Statins induce apoptosis and inhibit proliferation in cholangiocarcinoma cells. Int J Oncol. 2011;39(3): 561-8.
87. Furuya Y, Sekine Y, Kato H, Miyazawa Y, Koike H, Suzuki K. Low-density lipoprotein receptors play an important role in the inhibition of prostate cancer cell proliferation by statins. Prostate Int. 2016;4(2):56-60.
88. Chan J, Kulke M. Targeting the mTOR signaling pathway in neuroendocrine tumors. Curr Treat Options Oncol. 2014;15(3):365-79.
89. Memmott RM, Mercado JR, Maier CR, Kawabata S, Fox SD, Dennis PA. Metformin prevents tobacco carcinogen--induced lung tumorigenesis. Cancer Prev Res (Phila). 2010;3(9):1066-76.
90. Pelaia G, Gallelli L, Renda T, Fratto D, Falcone D, Caraglia M, et al. Effects of statins and farnesyl transferase inhibitors on ERK phosphorylation, apoptosis and cell viability in non-small lung cancer cells. Cell Prolif. 2012;45(6):557-65.
91. Vanova K, Boukalova S, Gbelcova H, Muchova L, Neuzil J, Gurlich R, et al. Heme oxygenase is not involved in the anti-proliferative effects of statins on pancreatic cancer cells. BMC cancer. 2016;16:309.
92. Garnett DJ, Greenhough TJ. Statins cause profound effects on gene expression in human cancer cells in vitro: the role of membrane microdomains. Gene Expr. 2012;15(5-6):225-34.
93. Nimako GK, Wintrob ZA, Sulik DA, Donato JL, Ceacareanu AC. Synergistic Benefit of Statin and Metformin in Gastrointestinal Malignancies. J Pharm Pract. 2017;30(2):185-94.
94. Del Campo JA, Garcia-Valdecasas M, Gil-Gomez A, Rojas A, Gallego P, Ampuero J, et al. Simvastatin and metformin inhibit cell growth in hepatitis C virus infected cells via mTOR increasing PTEN and autophagy. PLoS One. 2018;13(1):e0191805.
95. Babcook MA, Shukla S, Fu P, Vazquez EJ, Puchowicz MA, Molter JP, et al. Synergistic simvastatin and metformin combination chemotherapy for osseous metastatic castration-resistant prostate cancer. Mol Cancer Ther. 2014;13(10):2288-302.
96. Moreno A, Akcakanat A, Munsell MF, Soni A, Yao JC, Meric-Bernstam F. Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors. Endocr Relat Cancer. 2008;15(1):257-66.
97. Exner S, Prasad V, Wiedenmann B, Grotzinger C. Octreotide Does Not Inhibit Proliferation in Five Neuroendocrine Tumor Cell Lines. Front Endocrinol (Lausanne). 2018;9:146.

Chapter 9

General Discussion

Partially based on:
Neuroendocrine tumors: diagnostic, predictive and prognostic markers
Aura D Herrera-Martínez.[1,2], Leo J. Hofland[1], Wouter W. de Herder[1], María A. Gálvez Moreno[2], Justo P. Castaño[2] and Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.

Invited Review: Endocrine Related Cancer. Submitted for publication.

Medical Treatment for neuroendocrine tumors: current options and future perspectives
Aura D. Herrera-Martínez[1,2], Johannes Hofland[1], Leo J. Hofland[1], Tessa Brabander[3], Ferry A.L.M Eskens[4], María A. Gálvez Moreno[2], Justo P. Castaño[2], Wouter W. de Herder[1], Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.
[3] Department of Radiology & Nuclear Medicine, Erasmus Medical Center, Rotterdam, the Netherlands
[4] Department of Medical Oncology, Erasmus Medical Center, Rotterdam, the Netherlands

Invited Review: Drugs. Submitted for publication

GENERAL DISCUSSION

Neuroendocrine tumors (NETs) are a very heterogeneous group of neoplasms, which can present with a wide range of clinical symptoms and behavior. The possibility of recurrence or long-term metastasis adds to the complexity to the clinical management of patients with NET. Moreover, heterogeneity of NETs, in terms of clinical behavior and response to medical treatment, emphasizes the importance of identifying new diagnostic, prognostic and therapeutic markers [1].

Some biomarkers for functioning and non-functioning NETs are currently available. However, despite the application of these biomarkers in clinical practice, their results should be interpreted with caution [2]. Considering the variable sensitivity and specificity of these parameters, there is an unmet need for novel biomarkers to improve diagnosis and predict patient outcome. Nowadays, several new biomarkers are under study, and may become future tools for the management of NETs. These biomarkers include: 1) peptides and growth factors; 2) DNA and RNA markers based on genomic and transcriptomic analysis, including the so-called NET test, which has been developed for analyzing gene transcripts in circulating blood; 3) circulating tumor/ endothelial/progenitor cells or cell free tumor DNA, which represent non-invasive methods that may provide additional information for monitoring treatment response and tumor recurrence; and 4) improved imaging techniques with novel radiolabeled somatostatin analogs or peptides. Unfortunately, nowadays there is no consensus for supporting the use of these novel biomarkers as an indicator of tumor burden [3]. Furthermore, dysregulation of several biomarkers is not tumor specific, and the absence of cut-off levels for differentiating tissue and tumor subtypes, the lack of reproducibility in other NET cohorts and the difficulties in their interpretation, limit their clinical application. Thus, it is aimed to develop highly specific and sensitive circulating/tissue biomarkers using DNA, RNA and metabolomic approaches.

Despite the rising incidence of NETs in the last years, prognosis has improved, probably due to the increased detection of early stage of disease and the availability of novel therapeutic options. Treatment of patients with unresectable NETs is complex and aims to control hormone secretion and tumor growth. The development of next-generation multireceptor targeting and radiolabeled somatostatin analogs, as well as target-directed therapies (everolimus, sunitinib) improved progression-free survival (PFS) in NET patients. However, only a proportion of patients responds to these therapies. Validated biomarkers that predict response to target-directed therapies are not yet available, whereas the expression of somatostatin receptors (SSTs) in NETs can be used to select patients that are eligible for peptide receptor radionuclide therapy (PRRT). Therefore, there is a need to develop biomarkers that can be used to achieve a more personalized treatment approach in patients with NET.

Medical treatment with somatostatin analogs (SSAs) is well-known in differentiated, locally advanced or metastatic NETs [4]. SSAs act through binding to G-protein-coupled SSTs

which are broadly expressed in NETs and may modulate tumor cell proliferation and hormone secretion [5, 6]. Despite their efficacy in disease stabilization and symptom control, SSAs cannot control hormone production and tumor growth in the long term in many patients. For this reason, novel therapeutic options have been developed, such as PRRT [7], and specific pathways involved in cell proliferation, migration and/or angiogenesis in NETs have been targeted [8, 9]. In addition, chemotherapy schemes have been updated [10], new therapeutic options for functioning NETs have been developed, and several novel treatments are currently under study.

Based on the above-mentioned considerations, this thesis evaluated, on the one hand, the putative role of somatostatin and ghrelin system components as tissue (bio-)markers in NETs, and on the other hand, the antitumor effect of registered drugs for other medical purposes (ketoconazole, biguanides and statins), as well as the effect of novel drugs for hormone release control (somatostatin-dopamine receptor chimeras and telotristat) in NETs. For these aims, we used clinical data and cell-based assays, which represent a valuable, simple, and cost-effective tool for drugs discovery and evaluation [11]. Specifically, we used traditional two-dimensional (2D) monolayer cell cultures, in some studies, we completed our results with three-dimensional (3D) cell culture models using spheroids. This last culture system is a novel promising method which seems to appropriately reproduce tumor cells environment including, cell-cell signaling, growth kinetics, extracellular matrix deposition, nutrients-oxygen conditions, gene expression, drug resistance, cell heterogeneity and cell-cell physical interactions [12]. It has been described that 3D models with spheroids would allow better evaluation of long-term treatment [13-16], especially in NET cell lines [17]. According the results presented in this thesis, 3D cultures allowed studies with a reliable measure of hormone secretion in serum-deprived conditions, whereas an increased apoptosis, lactate dehydrogenase levels and decreased cell proliferation rates characterized long incubation periods (7 days) in 2D monolayer cell cultures. In this context, 3D culture systems with spheroids were especially useful in serum-deprived conditions, since they permit longer incubation periods with optimal (time-dependent) cell growth.

NET and somatostatin system

It has been widely described that the somatostatin system is linked to several intracellular signaling pathways including the regulation of cell proliferation, differentiation, and angiogenesis, amongst others [18]. Despite the fact that the presence of the somatostatin system has been widely described in NETs [19, 20], a specific role in tumorigenesis has not been fully elucidated yet [20, 21]. This includes the use for tumoral scintigraphy [22] and as target for long acting SSA and peptide receptor radionuclide therapy (PRRT) [22-24]. Recent publications have suggested that somatostatin system dysregulation may be associated with specific clinical features and prognosis in NETs patients [21, 25]. As such its molecular and immunohistochemical evaluation may represent a valuable prognostic marker in NETs [25, 26].

In the studies described in **chapters 2 and 3** of this thesis, we evaluated the mRNA expression of specific somatostatin system components, including the ligands somatostatin, cortistatin, and their receptors, SST_1, SST_2, SST_3, SST_4, SST_5 and SST_5TMD_4. Comparisons were made with normal (control), non-tumor adjacent (NTA) and tumor tissues. The presence of somatostatin system components was different between normal lung and gastroenteropancreatic (GEP) tissue. Specifically, in normal lung tissue, all somatostatin system components except somatostatin and SST_3 were expressed in less than 25% of samples, whereas in GEP-tissue, all system components (except cortistatin and SST_4) were expressed in more than 60% of cases. Additionally, the expression profile was also different among both groups: in lung carcinoids the mRNA expression of somatostatin and SST_4 was higher in tumor tissues compared to normal samples, while SST_5 was overexpressed in tumor samples compared to NTA. In contrast, in GEP-NETs, cortistatin, SST_1, SST_2, SST_5 were clearly over- expressed in tumor tissues compared with NTA samples.

As described in **chapters 2 and 3** of this thesis, differences in the expression of somatostatin system components in control (normal) and NTA tissue, as well as the correlation between the molecular expression of somatostatin and its receptors suggests an autocrine/paracrine effect that may modulate *in situ* tumor progression of NETs [27]. Additionally, interactions with other family receptors (opioids, dopamine, chemokine) have been described and may be associated with pathway activations [28], and even with tumor grade and malignancy [29].

According to our results, the different molecular expression of somatostatin system components in NTA tissue compared to normal samples (in lung carcinoids and in GEP-NETs) suggest that they may exert a biologically regulatory role in proliferation/secretion of these tumors. Among them, in GEP-NETs the overexpression of somatostatin was associated with increased lymphatic invasion but not with liver metastasis, additionally, SST_5 was associated with vascular and nerve invasion. In addition, other authors have suggested an association between its truncated isoform (SST_5TMD_4) with increased tumor aggressiveness, suggesting that a dysregulation in this receptor could be related to aggressive features and patient outcome [21, 30]. In contrast, in lung carcinoids, no relationship between SST_5 and agressiveness/invasion parameters was observed, while the truncated receptor SST_5TMD_4 was positively associated with disease free status during the follow-up. These results confirm the intrinsic variability and heterogeneity of NETs, specially in those with different localization, suggesting that an appropiate characterization of the tumor may allow a personalized clinical management. In this sense, not only the routine evaluation of SST_2 provides clinical information, but probably in the future the determination of other receptors, specially SST_5, may offer additional data about clinical outcome and prognosis.

As previously mentioned, SSTs expression in NETs is fundamental for the therapeutic management of these tumors. SSAs are first-line therapy in functionally active NETs, including those associated with the carcinoid syndrome and functional pancreatic neuroendocrine

tumors (PNETs) [31]. Both long-acting octreotide and lanreotide, which target the SST_2, similarly improve symptoms and quality of life in NET patients [32]. Unfortunately, some patients may be resistant to octreotide or lanreotide. For this reason, a novel SSA (pasireotide), which has high affinity to multiple SSTs, including SST_2 and SST_5, may represent a valuable therapeutic option due to its higher affinity to more SSTs [33], and is suggested to be of value in insulinomas resistant to other treatment options [34, 35]. In concordance to this, in **chapter 5 and 7** a not yet described *in vitro* inhibitory effect of pasireotide on serotonin release in PNET cell lines is shown, which may be related to the SST_5 expression in this cell line. This observation further enhances the importance of the molecular study of somatostatin system components in tumor samples of NETs. Importantly, the use of radiolabeled SSAs forms in PRRT has been shown as a successful therapeutic alternative in metastatic functioning NETs with SSTs expression. Specifically, therapy using peptide receptor radionuclide therapy (PRRT) in GEP-NETs is associated with response rates (18-44% by RECIST criteria), improvement in quality of life [36, 37], reduction in risk of progression or death (79%) and increased overall response rate (ORR; 18%) [38]. Despite PRRT is a promising treatment in GEP-NETs, the application in lung NETs may be limited due to a lower expression of SSTs, especially in some atypical lung carcinoids; notwithstanding, ORR of 15% and disease stabilization of 47% have been reported [39, 40], which is related to the findings observed in our cohort, in which SST_2 was expressed in NTA and tumor samples. Interestingly, clinical effects have been described even when tumor progression was observed after initial good response, suggesting that not only the antitumor effect of PRRT may be involved in the clinical response [41].

NETs and the Ghrelin system

Tightly associated with the somatostatin system, ghrelin system is also involved in the regulation of multiple (patho)-physiological functions, including hormonal secretion, β-cell survival or appetite and gastric motility (74, 75). Alterations in the expression of specific components of this system have been associated with the development/progression of various neoplasms, but these associations are still controversial and remain unclear (76-79).

According to our results, the differences in the expression of ghrelin system components between normal tissue, non-tumor adjacent tissue and tumor tissue, suggest a dysregulation of ghrelin system in these tumors Additionally, the concomitant expression of ghrelin, its activating enzyme ghrelin-O-acyl-transferase (GOAT) and the native receptor GHSR1a suggests that the whole machinery of this system may modulate the development or progression in this pathology, and may include some splicing variants (e.g. In1-ghrelin and GHSR1b) (29).

In this context, ghrelin system components were expressed in < 25% of normal lung samples, as determined by qPCR, but ghrelin, In1-ghrelin, GHSR1a and GHSR1b were expressed in at least 75% of lung carcinoids and NTA tissues. Specifically, GHSR1a and

GHSR1b were overexpressed in tumor tissue and NTA tissue compared to normal lung tissue. Interestingly, while the mRNA expression of ghrelin was associated with decreased vascular invasion capacity, the canonical receptor GHSR1a was associated with increased proportion of metastasis, suggesting that other unknown ligands may also bind this receptor. In contrast, in GEP-NETs, ghrelin and its native receptor GHSR1a were expressed in more than 75% of healthy controls, additionally, their expression levels were decreased in NTA and tumor tissue compared with normal samples, with a slightly, but not significantly, increased expression in tumor compared with NTA tissue. In these tumors, GHSR1a was associated with functioning tumors and decreased mortality, contrasting with the clinical findings observed in lung carcinoids.

An important finding of this thesis is the potential role of GOAT enzyme as biomarker in NETs. Specifically, GOAT acylates the third serine residue of ghrelin. After this unique modification, acylated ghrelin represents the peptide binding and activating its canonical ghrelin receptor GHSR1a (80, 81). In lung carcinoids, GOAT was virtually absent in control tissue, whereas it was present in less than 50% of the NTA samples and raised to more than 75% of tumor samples, despite this, it was not overexpressed in tumor tissue compared to NTA. In contrast, in GEP-NETs, GOAT was detected in about 20% of normal samples and was remarkably overexpressed in tumor tissues compared with NTA regions and normal tissues, especially in gastrointestinal NETs, compared to pancreatic NETs. Despite GOAT was not associated with any invasion/aggressiveness parameter, its remarkable overexpression in tumor tissue suggests a potential role in NETs, further research is required and would probably include its determination as circulating marker, or even more, the evaluation of its role as a therapeutic marker by inducing changes in its molecular expression or function. Although clinical results presented in this thesis have been evaluated in ample, well-characterized patient cohorts, the heterogeneity of the included patients and the retrospective analysis, may limit their reproducibility. Despite this, results presented in Part I of this thesis, provide a bases for future characterization and study of these system components in NETs, including e.g. circulating markers analysis in prospective cohorts.

Novel targeted therapies

The availability of molecular-target therapies in NETs has increased the therapeutic alternatives for NETs. Sunitinib and everolimus have been described as successful treatment options in both functioning and non-functioning tumors, since they may control tumor growth, improve clinical symptoms and increase PFS [42, 43]. Unfortunately, significant prolongation of overall survival (OS) with these medical options is still controversial and novel therapeutic options, especially for hormone release control, are necessary.

Somatostatin-dopamine chimeric drugs

Multi-receptor interaction has been suggested as an efficacious and selective therapeutic strategy for enhancing the effects of somatostatin [28]. It has been hypothesized that this modulation may be achieved by the activation of different receptor systems with a common endpoint, an increased activation of a single/common pathway by multiple receptors, or the activation of multiple complimentary intracellular signal transduction systems [28]. The presence of hetero-dimers has been described among SSTs and between SSTs and other receptor families, including dopamine receptors, especially the dopamine receptor type 2 (D2R) [44, 45]. Based on this, some structural chimeric molecules that combine elements of SSAs and dopamine agonists (DA) were developed [28]. The potential effect of these chimeras depends on the affinity for each receptor subtype, which may be modified according to the therapeutic interest. An ideal chimeric compound would have potent SST_2 and D2R activity, with moderate SST_5 activity in order to inhibit hormone secretion without altering the glucose homeostasis [28, 46]. In **chapter 6**, the effect on cell proliferation and secretion of two SSTs-D2R multi receptor targeting drugs (BIM065 and BIM23A760) is described using two-dimensional (2D; monolayer) and three-dimensional (3D; spheroids) culture systems. Their inhibitory effect on serotonin and CgA secretion, which was comparable to cabergoline but not to octreotide, suggests that in this PNET model, the activity of both chimeric drugs is probably related to their affinity for D2R. *In vivo* reports have described that BIM-23A760 acutely decreases growth hormone and prolactin secretion in pituitary tumors, but long-term effects disappeared due to a dopaminergic metabolite that may interfere with the activity of the parent molecule [28]. In our study, BIM-23A760, followed by cabergoline and BIM-065, were the most potent drugs for inhibiting serotonin and CgA secretion. In NETs, an open label, multicenter clinical trial in patients with carcinoid syndrome was started for evaluating the efficacy of BIM-23A760. This study was prematurely terminated, and unfortunately, primary/secondary outcomes were not analyzed (NCT01018953). Due to their putative promising effects, current research is focused in the improvement of chimeric molecules that could keep a long-term effect. The results described in **chapter 6** also underline that it is important to recognize the effect of cabergoline in hormone release control in NETs, in addition to that of SSA. This is further strengthened with previous reports of the use of cabergoline in ectopic ACTH-producing NETs (monotherapy or in combination with lanreotide) when D2R expression is observed in the tumor [47-49].

Telotristat

Interestingly, the novel inhibitor of tryptophan hydroxylase, telotristat has been described as an effective therapeutic option for serotonin release control [50]. The use of telotristat has been recently approved by the European Commission and the Food and Drug Administration [51]. Decreased urinary 5-hydroxyindoleacetic acid and small bowel movements were reported in the TELESTAR [52] and TELECAST phase III clinical trials [53]. Surprisingly,

despite its clinical use, to the best of our knowledge no pre-clinical data exist in which the *in vitro* effect of this novel drug in NET has been tested. In this sense, the first report of the *in vitro* effects of telotristat is presented in **chapter 7** of this thesis. According to our *in vitro* model, a dose-dependent decreased serotonin release was observed. Importantly, the effects were achieved with clinically relevant concentrations, without concomitant cytotoxic effects or changes in the molecular expression of SSTs. Remarkably, serotonin has been described as a potent mitogen in different cell types [54]. However, in the evaluated pancreas NET 3D culture model, no effect on cell growth was observed, whereas serotonin secretion was totally suppressed. These data suggest that these cells may not respond to a direct paracrine/autocrine effect of serotonin on cell proliferation. On the other hand, serotonin might influence tumor growth via an interaction with the tumor microenvironment as well. Finally, we unexpectedly observed a slightly decreased effectiveness of the combination therapy with octreotide in BON-1 cells. This finding requires further investigation because of its implication in the clinical practice.

Importantly, in the studies presented in chapter 6 and 7 of this thesis, we used a PNET model for studying serotonin secretion in NETs, whereas an ideal model might be a midgut cell line. Unfortunately, midgut NET cell lines are not widely available. A recent study showed that of seven established NET cell lines, only the small intestine NET lines GOT-1, P-STS and the PNET lines BON-1 and QGP-1 display a neuroendocrine phenotype, as well as disease-characteristic mutations. Other cell lines were identified as lymphoblastoid (e.g. KRJ-1) [55]. Unfortunately, the GOT-1 and P-STS cell lines were not available in our laboratory to confirm our findings in a midgut-NET model. Finally, it will be important to evaluate the effects of SSTs/D2R chimeric drugs, as well as telotristat, in NET primary cultures.

Ketoconazole

It is important to recognize that some drugs that are prescribed for other medical purposes may have some direct or indirect effects for tumor control. Their combination with SSAs would represent a valuable method for increasing the antitumor effects or even to control hormone secretion. In this sense, previous reports of the cytotoxic effect of ketoconazole in cancer [56] were confirmed in **chapter 5** using a lung- and pancreas model of adrenocorticotropin- (ACTH)-producing and non-ACTH producing NET cells, respectively. In these two NET models, ketoconazole may exert its action following different mechanisms including apoptosis induction, cell toxicity and decreased cell division. The cell growth inhibitory effects observed in our study suggest that a direct antitumor action of ketoconazole may be involved in the sustained effect achieved with this drug [57]. Despite some effects of ketoconazole were reached with clinically relevant concentrations, its use in non-ACTH producing tumors is controversial and requires further studies.

Metformin and statins

It is well-known that the inflammation and insulin resistance observed in patients with type 2 diabetes (T2-DM) or metabolic syndrome are associated with increased incidence of neoplasms [58]. Thus, targeting related pathways may represent appropriate therapeutic options in some types of cancer. Based on this, the putative antitumor effects of biguanides and statins in NET were described in **chapter 8**. Biguanides stimulate AMP-activated protein kinase (AMPK), which induces cell cycle arrest, reduces the insulin/insulin growth factor 1 (IGF-1) signaling [59, 60] and, additionally, suppresses the mammalian target of rapamycin (mTOR1) which is a key regulator of cell proliferation in cancer cells [61, 62]. For this reason, the biguanide metformin has been evaluated in multiple *in vitro* and *in vivo* studies [63] and is now being tested in registered clinical trials in NETs in combination with lanreotide (NCT02823691) in well-differentiated gastrointestinal or lung NETs. Interestingly, those clinical variables that in our cohort were related to metabolic syndrome were reversed when treated with metformin. In addition, the inverse expression of somatostatin and ghrelin systems when diabetic patients were treated with metformin, suggests that this drug may exert an additive effect for tumor control since it improves the expression of SSTs and may therefore potentiate the effects of SSAs. Although preliminary, these results are encouraging and allow to hypothesize that the effect of metformin, as an adjuvant therapy to classic therapeutic regimens, should be evaluated in appropriately designed prospective clinical studies.

Statins may also have additional anti-tumor effects, including induced cell-cycle arrest, apoptosis induction, decreased invasion/metastasis capacity and decreased Ki67 expression [64-68]. Our *in vitro* results are concordant with previous reports of the anti-proliferative effect of these drugs [69-71]. Despite this, in our cohort of patients, we did not observe a statistically significant effect of statins on clinical features of NET patients, but a higher proportion of disease-free patients was observed in this group. Importantly, the size of the cohort may have affected the significance of the results and unfortunately, there are no clinical data available for comparing the effects of statins in NETs. To the best of our knowledge, no clinical trial for evaluating the effects of statins in NETs is currently registered.

Other future directions for diagnostic markers and treatment in NETs

Current circulating biomarkers for NETs have several limitations, especially in terms of sensitivity, specificity and reproducibility. To date, several prospective trials are evaluating the effect of novel therapeutic strategies in NETs (e.g. 90Y, 177Lu, Tyr octreotate, lenvatinib, palbociclib, tremelimumab, bevacizumab, temozolamide, pasireotide, PDR001), and most of them include the evaluation of treatment-related follow-up markers. Despite the advances in NET biomarkers in recent years, it is not possible yet to accurately predict treatment response and patient outcome. Additionally, the intrinsic heterogeneity of NETs, their variable behavior and prognosis complicates the identification of specific and reliable biomarkers to predict medical treatment response and patient prognosis.

Nowadays, several biomarkers for NETs are under study. Probably multianalyte measurements based on genomics represent the most accurate system for early outcome stratification and decision making. The development of blood-based analysis and liquid biopsy may represent non-invasive methods for diagnosis and prognosis as well. Imaging techniques may also improve when combined with molecular markers, not only radiolabeled SSAs, but also peptides, including the combination of glucose transporters/positron emission tomography for *in vivo* imaging. Circulating tumor cells, miRNAs, cytokines and gene transcripts represent putative novel biomarkers that could predict clinical outcome and response to treatment. Genomics will probably become the basis for developing multitranscript biomarkers, their combination would provide multifaceted information, may offer better medical management and may improve the use of resources in order to improve diagnosis, treatment, quality of life and survival in NET patients [1].

Medical treatment options in NETs have significantly increased and improved in the last years. Despite this, some patients may not respond or develop treatment resistance. In these cases, more therapeutic options that allow a personalized management would be useful. A higher number of clinical trials and approved therapeutic agents facilitate the management of patients. As a consequence, a treatment sequence should be established taken into account response rate, improvements in progression-free survival and overall survival. To this aim, head to head studies would help to identify the best therapeutic options in specific cases. Importantly, overall survival in NETs has improved despite an increased incidence of these tumors, which may be related to the advances in tumor diagnosis and treatment. However, tumor heterogeneity as well as several aspects of the biologic, genetic and molecular background in NETs are still unknown and require further investigation. For this reason, it is needed to determine novel therapeutic targets that could improve the medical management of hormone-related syndromes and the antiproliferative treatment in NETs. Novel medical options and treatment combinations will be available in the upcoming years. Although preliminary results of clinical trials and *in vitro* models are encouraging, large longitudinal randomized studies are still required to provide accurate evidence of their effect on survival and symptom control.

Targeting signaling and immune pathways involved in NETs development and progression has provided novel therapeutic options for these tumors. Immunotherapy is probably one of the most interesting fields that will be evaluated in NETs in the upcoming years. Despite immunotherapy has an important role in the management of other types of cancer, the effect on well-differentiated NETs according to preliminary data seems to be limited, but it may have a role in neuroendocrine carcinomas. Several clinical trials in this field are ongoing and hopefully in the upcoming years, some results about their real clinical applicability in NETs will be available.

REFERENCES

1. Modlin, I.M., et al., *Neuroendocrine tumor biomarkers: current status and perspectives.* Neuroendocrinology, 2014. **100**(4): p. 265-77.
2. Oberg, K., et al., *Consensus on biomarkers for neuroendocrine tumour disease.* Lancet Oncol, 2015. **16**(9): p. e435-e446.
3. Oberg, K., et al., *A Delphic consensus assessment: imaging and biomarkers in gastroenteropancreatic neuroendocrine tumor disease management.* Endocr Connect, 2016. **5**(5): p. 174-87.
4. de Herder, W.W., A.J. van der Lely, and S.W. Lamberts, *Somatostatin analogue treatment of neuroendocrine tumours.* Postgrad Med J, 1996. **72**(849): p. 403-8.
5. Wangberg, B., et al., *[Somatostatin receptors. A new way to diagnosis and therapy of neuroendocrine tumors].* Lakartidningen, 1997. **94**(10): p. 829-30, 835-8.
6. Cakir, M., D. Dworakowska, and A. Grossman, *Somatostatin receptor biology in neuroendocrine and pituitary tumours: part 2--clinical implications.* J Cell Mol Med, 2010. **14**(11): p. 2585-91.
7. van der Zwan, W.A., et al., *GEPNETs update: Radionuclide therapy in neuroendocrine tumors.* Eur J Endocrinol, 2015. **172**(1): p. R1-8.
8. Raymond, E., et al., *Therapy innovations: tyrosine kinase inhibitors for the treatment of pancreatic neuroendocrine tumors.* Cancer Metastasis Rev, 2011. **30 Suppl 1**: p. 19-26.
9. Chan, J. and M. Kulke, *Targeting the mTOR signaling pathway in neuroendocrine tumors.* Curr Treat Options Oncol, 2014. **15**(3): p. 365-79.
10. Bongiovanni, A., et al., *First-line chemotherapy in patients with metastatic gastroenteropancreatic neuroendocrine carcinoma.* Onco Targets Ther, 2015. **8**: p. 3613-9.
11. Edmondson, R., et al., *Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors.* Assay Drug Dev Technol, 2014. **12**(4): p. 207-18.
12. Costa, E.C., et al., *3D tumor spheroids: an overview on the tools and techniques used for their analysis.* Biotechnol Adv, 2016. **34**(8): p. 1427-1441.
13. Baker, B.M. and C.S. Chen, *Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues.* J Cell Sci, 2012. **125**(Pt 13): p. 3015-24.
14. Harma, V., et al., *A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses.* PLoS One, 2010. **5**(5): p. e10431.
15. Kimlin, L.C., G. Casagrande, and V.M. Virador, *In vitro three-dimensional (3D) models in cancer research: an update.* Mol Carcinog, 2013. **52**(3): p. 167-82.
16. Kondo, J., et al., *Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer.* Proc Natl Acad Sci U S A, 2011. **108**(15): p. 6235-40.
17. Wong, C., et al., *Human neuroendocrine tumor cell lines as a three-dimensional model for the study of human neuroendocrine tumor therapy.* J Vis Exp, 2012(66): p. e4218.
18. Volante, M., F. Bozzalla-Cassione, and M. Papotti, *Somatostatin receptors and their interest in diagnostic pathology.* Endocr Pathol, 2004. **15**(4): p. 275-91.
19. Kwekkeboom, D.J., et al., *ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: somatostatin receptor imaging with (111)In-pentetreotide.* Neuroendocrinology, 2009. **90**(2): p. 184-9.
20. Kanakis, G., et al., *Expression of Somatostatin Receptors 1-5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role.* Neuroendocrinology, 2015. **101**(3): p. 211-22.

21. Sampedro-Nunez, M., et al., *Presence of sst5TMD4, a truncated splice variant of the somatostatin receptor subtype 5, is associated to features of increased aggressiveness in pancreatic neuroendocrine tumors.* Oncotarget, 2016. **7**(6): p. 6593-608.
22. Lamberts, S.W., J.C. Reubi, and E.P. Krenning, *Somatostatin receptor imaging in the diagnosis and treatment of neuroendocrine tumors.* J Steroid Biochem Mol Biol, 1992. **43**(1-3): p. 185-8.
23. Eriksson, B., et al., *The use of new somatostatin analogues, lanreotide and octastatin, in neuroendocrine gastro-intestinal tumours.* Digestion, 1996. **57 Suppl 1**: p. 77-80.
24. Brabander, T., et al., *Peptide receptor radionuclide therapy of neuroendocrine tumours.* Best Pract Res Clin Endocrinol Metab, 2016. **30**(1): p. 103-14.
25. Kaemmerer, D., et al., *Somatostatin receptors in bronchopulmonary neuroendocrine neoplasms: new diagnostic, prognostic, and therapeutic markers.* J Clin Endocrinol Metab, 2015. **100**(3): p. 831-40.
26. Mizutani, G., et al., *Expression of Somatostatin Receptor (SSTR) Subtypes (SSTR-1, 2A, 3, 4 and 5) in Neuroendocrine Tumors Using Real-time RT-PCR Method and Immunohistochemistry.* Acta Histochem Cytochem, 2012. **45**(3): p. 167-76.
27. Herrera-Martinez, A.D., et al., *The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features.* Lung Cancer, 2017. **109**: p. 128-136.
28. Culler, M.D., *Somatostatin-dopamine chimeras: a novel approach to treatment of neuroendocrine tumors.* Horm Metab Res, 2011. **43**(12): p. 854-7.
29. Kaemmerer, D., et al., *Inverse expression of somatostatin and CXCR4 chemokine receptors in gastroenteropancreatic neuroendocrine neoplasms of different malignancy.* Oncotarget, 2015. **6**(29): p. 27566-79.
30. Herrera-Martinez, A.D., et al., *Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors.* Endocrine, 2018. **59**(2): p. 426-437.
31. Pavel, M., et al., *ENETS Consensus Guidelines Update for the Management of Distant Metastatic Disease of Intestinal, Pancreatic, Bronchial Neuroendocrine Neoplasms (NEN) and NEN of Unknown Primary Site.* Neuroendocrinology, 2016. **103**(2): p. 172-85.
32. O'Toole, D., et al., *Treatment of carcinoid syndrome: a prospective crossover evaluation of lanreotide versus octreotide in terms of efficacy, patient acceptability, and tolerance.* Cancer, 2000. **88**(4): p. 770-6.
33. Kvols, L.K., et al., *Pasireotide (SOM230) shows efficacy and tolerability in the treatment of patients with advanced neuroendocrine tumors refractory or resistant to octreotide LAR: results from a phase II study.* Endocr Relat Cancer, 2012. **19**(5): p. 657-66.
34. Tirosh, A., et al., *Pasireotide for malignant insulinoma.* Hormones (Athens), 2016. **15**(2): p. 271-6.
35. Hendren, N.S., et al., *Pasireotide for the treatment of refractory hypoglycaemia from malignant insulinoma.* Clin Endocrinol (Oxf), 2017.
36. Kim, S.J., et al., *The efficacy of (177)Lu-labelled peptide receptor radionuclide therapy in patients with neuroendocrine tumours: a meta-analysis.* Eur J Nucl Med Mol Imaging, 2015. **42**(13): p. 1964-70.
37. Khan, S., et al., *Quality of life in 265 patients with gastroenteropancreatic or bronchial neuroendocrine tumors treated with [177Lu-DOTA0,Tyr3]octreotate.* J Nucl Med, 2011. **52**(9): p. 1361-8.
38. Strosberg, J., et al., *Phase 3 Trial of (177)Lu-Dotatate for Midgut Neuroendocrine Tumors.* N Engl J Med, 2017. **376**(2): p. 125-135.

39. Ianniello, A., et al., *Peptide receptor radionuclide therapy with (177)Lu-DOTATATE in advanced bronchial carcinoids: prognostic role of thyroid transcription factor 1 and (18)F-FDG PET.* Eur J Nucl Med Mol Imaging, 2016. **43**(6): p. 1040-6.
40. Brabander, T., et al., *Long-Term Efficacy, Survival, and Safety of [(177)Lu-DOTA(0),Tyr(3)] octreotate in Patients with Gastroenteropancreatic and Bronchial Neuroendocrine Tumors.* Clin Cancer Res, 2017. **23**(16): p. 4617-4624.
41. de Herder, W.W., et al., *New therapeutic options for metastatic malignant insulinomas.* Clin Endocrinol (Oxf), 2011. **75**(3): p. 277-84.
42. Raymond, E., et al., *Sunitinib malate for the treatment of pancreatic neuroendocrine tumors.* N Engl J Med, 2011. **364**(6): p. 501-13.
43. Yao, J.C., et al., *Everolimus for advanced pancreatic neuroendocrine tumors.* N Engl J Med, 2011. **364**(6): p. 514-23.
44. Rocheville, M., et al., *Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers.* J Biol Chem, 2000. **275**(11): p. 7862-9.
45. Rocheville, M., et al., *Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity.* Science, 2000. **288**(5463): p. 154-7.
46. Jaquet, P., et al., *Efficacy of chimeric molecules directed towards multiple somatostatin and dopamine receptors on inhibition of GH and prolactin secretion from GH-secreting pituitary adenomas classified as partially responsive to somatostatin analog therapy.* Eur J Endocrinol, 2005. **153**(1): p. 135-41.
47. Pivonello, R., et al., *Dopamine receptor expression and function in corticotroph ectopic tumors.* J Clin Endocrinol Metab, 2007. **92**(1): p. 65-9.
48. Pivonello, R., et al., *Cabergoline plus lanreotide for ectopic Cushing's syndrome.* N Engl J Med, 2005. **352**(23): p. 2457-8.
49. Sakihara, S., et al., *A case of ectopic ACTH syndrome treated with intermittent administration of dopamine agonists.* Endocrinol Diabetes Metab Case Rep, 2014. **2014**: p. 140001.
50. Pavel, M., et al., *Telotristat ethyl in carcinoid syndrome: safety and efficacy in the TELECAST phase 3 trial.* Endocr Relat Cancer, 2018. **25**(3): p. 309-322.
51. 2017, F.N.R., *FDA Approves Xermelo for Carcinoid Syndrome Diarrhea. Silver Springs, M., USA: FDA. (available at: https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm544035.htm).*
52. Kulke, M.H., et al., *Telotristat Ethyl, a Tryptophan Hydroxylase Inhibitor for the Treatment of Carcinoid Syndrome.* J Clin Oncol, 2017. **35**(1): p. 14-23.
53. Pavel, M., et al., *Telotristat etiprate for carcinoid syndrome: a single-arm, multicenter trial.* J Clin Endocrinol Metab, 2015. **100**(4): p. 1511-9.
54. Fanburg, B.L. and S.L. Lee, *A new role for an old molecule: serotonin as a mitogen.* Am J Physiol, 1997. **272**(5 Pt 1): p. L795-806.
55. Hofving, T., et al., *The neuroendocrine phenotype, genomic profile and therapeutic sensitivity of GEPNET cell lines.* Endocr Relat Cancer, 2018. **25**(3): p. 367-380.
56. Rochlitz, C.F., et al., *Cytotoxicity of ketoconazole in malignant cell lines.* Cancer Chemother Pharmacol, 1988. **21**(4): p. 319-22.
57. Sharma, S.T. and L.K. Nieman, *Prolonged remission after long-term treatment with steroidogenesis inhibitors in Cushing's syndrome caused by ectopic ACTH secretion.* Eur J Endocrinol, 2012. **166**(3): p. 531-6.
58. Braun, S., K. Bitton-Worms, and D. LeRoith, *The link between the metabolic syndrome and cancer.* Int J Biol Sci, 2011. **7**(7): p. 1003-15.

59. Jalving, M., et al., *Metformin: taking away the candy for cancer?* Eur J Cancer, 2010. **46**(13): p. 2369-80.
60. Micic, D., et al., *Metformin: its emerging role in oncology.* Hormones (Athens), 2011. **10**(1): p. 5-15.
61. Zhou, G., et al., *Role of AMP-activated protein kinase in mechanism of metformin action.* J Clin Invest, 2001. **108**(8): p. 1167-74.
62. Hawley, S.A., et al., *Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation.* Cell Metab, 2010. **11**(6): p. 554-65.
63. Kourelis, T.V. and R.D. Siegel, *Metformin and cancer: new applications for an old drug.* Med Oncol, 2012. **29**(2): p. 1314-27.
64. Denoyelle, C., et al., *Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study.* Carcinogenesis, 2001. **22**(8): p. 1139-48.
65. Brozovic, A., et al., *Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance.* Int J Cancer, 2004. **112**(6): p. 974-85.
66. Koyuturk, M., M. Ersoz, and N. Altiok, *Simvastatin induces apoptosis in human breast cancer cells: p53 and estrogen receptor independent pathway requiring signalling through JNK.* Cancer Lett, 2007. **250**(2): p. 220-8.
67. Garwood, E.R., et al., *Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer.* Breast Cancer Res Treat, 2010. **119**(1): p. 137-44.
68. Bjarnadottir, O., et al., *Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial.* Breast Cancer Res Treat, 2013. **138**(2): p. 499-508.
69. Crescencio, M.E., et al., *Statins inhibit the proliferation and induce cell death of human papilloma virus positive and negative cervical cancer cells.* Int J Biomed Sci, 2009. **5**(4): p. 411-20.
70. Crosbie, J., et al., *Statins inhibit proliferation and cytotoxicity of a human leukemic natural killer cell line.* Biomark Res, 2013. **1**(1): p. 33.
71. Feldt, M., et al., *Statin-induced anti-proliferative effects via cyclin D1 and p27 in a window-of-opportunity breast cancer trial.* J Transl Med, 2015. **13**: p. 133.

Chapter 10

Summary

Samenvatting

Resumen

SUMMARY

Neuroendocrine tumors (NETs) represent a heterogeneous group of rare, slow-growing neoplasms, originating from enterochromaffin cells. Their annual incidence has progressively increased, although it is not known whether this is a true increase in NET incidence, the result of increased use of (improved) diagnostic procedures, or a combination of both. While metastatic disease is frequently observed at diagnosis, survival in NET patients has increased, which may be related to the advances in diagnostic and therapeutic procedures. NETs require appropriate standardized diagnostic procedures to assure early diagnosis, monitor disease progression and guide an optimal treatment. Additionally, medical treatment options in NETs have significantly increased and improved in the last years. This thesis evaluates a number of novel diagnostic and therapeutic options for patients with NET.

Chapter 1 provides a general overview about NETs, emphasizes the applicability and limitations of the current diagnostic biomarkers and summarizes some putative novel biomarkers in NETs. At the same time, this chapter describes current and novel therapeutic options for functioning a non-functioning NETs. The chapter introduces the main content of this thesis: novel diagnostic and therapeutic options for NETs. Chapter 1 ends with an overview of the main aims of this thesis.

Chapter 2 comprehensively evaluates the expression of several components of the somatostatin/cortistatin and ghrelin systems in a large series of well-characterized typical and atypical lung carcinoids. To the best of our knowledge, this study represents the first systematic characterization of the components of these regulatory systems in samples from lung carcinoids (LCs), in which a comparison is made with adjacent non-tumor regions and normal lung tissue. In this study, we observed that the expression of somatostatin, of some receptor subtypes (SSTs), and of the ghrelin receptor GHSR gradually increases from normal to non-tumoral adjacent and tumor tissue. Moreover, the presence of the truncated isoforms SST_5TMD_4, In1-ghrelin and the ghrelin receptor GHSR1b are first reported in LCs. Higher expression levels of ghrelin-O-acyltransferase (GOAT) were observed in tumors with necrosis, which were tumors with a larger size and higher capacity of peritumoral invasion and distant metastasis. These data suggest that a dysregulation of ghrelin system may be involved in the development/progression of these tumors.

Chapter 3 evaluates the expression of the somatostatin/cortistatin system components in gastroenteropancreatic- (GEP-) NETs. The observed overexpression of SST_5 in tumor tissue compared to adjacent non-tumor and normal tissue, as well as its clinical relation with vascular and nerve invasion suggests the putative role of SST_5 as a target for treating aggressive GEP-NETs. Furthermore, it emphasizes that the evaluation of SSTs profile in GEP-NETs may provide additional information for clinical decision making, including the use of second-generation somatostatin analogs (SSAs).

The most novel and relevant finding of **chapter 4** is the marked overexpression of GOAT in GEP-NET samples. Whereas the expression of this enzyme is almost absent in the corresponding normal tissues, it is present in adjacent non-tumoral tissue and notably overexpressed tumor tissues. Additionally, its overexpression was demonstrated using immunohistochemistry. Importantly, this enzyme was associated to larger tumors, especially in gastrointestinal-NETs. These findings, in combination with previous publications, suggest a putative role of GOAT as a diagnostic biomarker in GEP-NETs.

Chapter 5 evaluates the effect of ketoconazole on proliferation, cell cycle, apoptosis and secretion of adrenocorticotropic hormone (ACTH) or serotonin and chromogranin in an ACTH-producing bronchial NET and a non-ACTH producing pancreatic NET cell line, respectively. Ketoconazole exerted a predominant pro-apoptotic or cytotoxic effect, depending on the cell line. Additionally, ketoconazole induced changes in the cell cycle progression, e.g. it increases G0/G1 phase in both cell lines and an arrest in G2/M phase in the pancreatic NET cell line. Furthermore, a non-previously reported inhibitory effect of the SSA pasireotide on serotonin secretion by pancreatic NET cells was reported, which is probably related to the predominant SST_5 expression in this cell line.

In **chapter 6,** a comprehensive characterization of two pancreatic NET models was performed using two- (2D; monolayer) and three- dimensional (3D; spheroids) culture systems in different medium conditions. According to our results, NET 3D spheroid cultures represent a promising method for evaluating cell proliferation and secretion in NET cell lines, even in serum-deprived conditions. A non-previously reported dynamic expression of SSTs and dopamine receptor (D2R) was observed during growth of both 2D and 3D cultures. The effects of the somatostatin analog octreotide, the dopamine agonist cabergoline and of two novel SSTs-D2R multi-receptor targeting drugs (BIM-065, BIM-23A760) were evaluated using both culture systems. Cabergoline and SSTs-D2R multi-receptor targeting drugs, but not octreotide, inhibited CgA and serotonin secretion, but not NET cell growth. This suggests that the effect of the SSTs-D2R multi-receptor targeting drugs on secretion is mediated by D2R, and may indicate a putative role of dopamine agonists for treating D2R expressing functioning NETs, including carcinoid syndrome.

Chapter 7 shows the *in vitro* effects of the novel tryptophan hydoxylase inhibitor telotristat on pancreatic NET cells. Telotristat potently inhibited serotonin release in a dose-dependent manner at a clinically feasible concentration. Its combination with pasireotide, but not with octreotide, had an additive effect on serotonin secretion. In 3D cultured pancreatic NET cells, serotonin secretion was completely blocked by telotristat, whereas this drug did not influence cell growth. These data suggest that serotonin has no autocrine/paracrine effect on cell growth in this 3D PNET model.

In **chapter 8** decreased expression of SSTs was observed in tumor tissue of GEP-NET patients with type 2 diabetes, compared to non-diabetic GEP-NET patients. This decreased expression was not observed in type 2 diabetic patients that were treated with metformin.

The *in vitro* experiments showed an antiproliferative effect of biguanides and statins in NET cell lines, with consequent increased apoptosis and decreased cell migration. Since metformin and statins are low cost commercially available drugs, with a large experience in their clinical use, they may represent putative options for adjuvant therapy in NETs.

In **Chapter 9**, the results and conclusions of the studies included in this thesis, are discussed in relation to current and possible novel future diagnostic modalities, as well as therapeutic options for NET patients.

SAMENVATTING

Neuroendocriene tumoren (NET) vormen een heterogene groep van zeldzaam voorkomende, langzaam groeiende tumoren. NET ontstaan vanuit enterochromaffine cellen. De jaarlijkse incidentie van NET is progressief toegenomen in de afgelopen jaren. Het is echter niet duidelijk of deze toename in incidentie een werkelijke toename is, veroorzaakt wordt door verbeterde diagnostische procedures, of door een combinatie van beiden. Hoewel NET vaak al gemetastaseerd blijken te zijn bij de diagnose, is de gemiddelde overleving van patiënten met NET toegenomen. Dit is waarschijnlijk toe te schrijven aan verbeterde diagnostiek en toegenomen therapeutische mogelijkheden. Er zijn goede en gestandaardiseerde diagnostische procedures nodig om patiënten met een NET in een zo vroeg mogelijk stadium te diagnosticeren, om het beloop van de ziekte nauwlettend te vervolgen en om deze patiënten een optimale behandeling te kunnen geven. In de afgelopen jaren is het aantal medicamenteuze behandelingsmogelijkheden voor patiënten met een NET sterk toegenomen. In dit proefschrift worden een aantal studies beschreven die betrekking hebben op diagnostische en therapeutische mogelijkheden voor NET patiënten.

Hoofdstuk 1 betreft een algemene inleiding over NET en benadrukt de toepassing en beperkingen van de huidige diagnostische procedures voor patiënten met NET. Er wordt een overzicht gegeven van een aantal mogelijk nieuwe diagnostische (bio)merkers. In dit hoofdstuk worden tevens huidige en nieuwe behandelingsmogelijkheden voor functionele en niet-functionele NET besproken. Dit hoofdstuk vormt de inleiding voor het onderwerp van dit proefschrift, n.l. nieuwe diagnostische en therapeutische mogelijkheden voor patiënten met NET. Hoofdstuk 1 wordt afgesloten met een overzicht van de doelstellingen van het onderzoek van het proefschrift.

In het onderzoek beschreven in **hoofdstuk 2** is de expressie van verschillende componenten van het somatostatine/cortistatine en het ghrelin systeem onderzocht in een grote serie van goed gekarakteriseerde typische en atypische long carcinoiden (LC). Voor zover bekend is dit de eerste studie waarin beide systemen tegelijkertijd worden onderzocht in LC weefsels. Het onderzoek toont aan dat de expressie van somatostatine, van enkele somatostatine receptor subtypen (SSTs), en van de ghrelin receptor GHSR, gradueel toeneemt van normaal naar niet tumor-aangrenzend weefsel en tumor weefsel. Bovendien werd voor de eerste keer in LC expressie aangetoond van de korte iso-vormen SST5TMD4, In1-ghrelin en de ghrelin receptor GHSR1b. Hogere expressie van ghrelin-O-acetyltrasferase (GOAT) werd gevonden in tumoren met necrose, grotere tumoren met meer peritumorale invasie en metastasen op afstand. Deze bevindingen suggereren dat een ontregelde regulatie van het ghrelin systeem betrokken zou kunnen zijn bij de ontwikkeling/progressie van de tumoren.

In **hoofdstuk 3** wordt onderzoek naar de expressie van componenten van het somatostatine/cortistatine systeem in gastroenteropancreatische- (GEP-) NET beschreven. De gevonden hogere expressie van SST_5 in tumor weefsel in vergelijking met tumor-aangrenzend en met

normaal weefsel, en de klinische relatie van SST_5 expressie met vasculaire- en zenuw invasie, suggereert dat SST_5 een mogelijk doelwit is om agressieve GEP-NET te behandelen. Bovendien kan de evaluatie van het SSTs profiel in GEP-NET aanvullende informatie opleveren die mogelijk gebruikt kan worden bij klinische besluitvorming, waaronder het gebruik van een tweede generatie somatostatine analogen (SSA).

De meest nieuwe en relevante bevinding van het onderzoek beschreven in **hoofdstuk 4** is de duidelijke overexpressie van GOAT in GEP-NET weefsel. Terwijl de expressie van dit enzym vrijwel afwezig is in bijbehorend normaal weefsel, wordt expressie gevonden in aangrenzend niet-tumor weefsel en is expressie van GOAT met name aanwezig in tumor weefsel. De hogere expressie van GOAT in GEP-NET weefsel werd tevens aangetoond met behulp van immunohistochemie. Hoge expressie van dit enzym was geassocieerd met grotere tumoren en voornamelijk in gastro-intestinale NET. Deze bevindingen, tezamen met eerdere publicaties, suggereren dat GOAT een mogelijke diagnostische biomerker is in GEP-NET.

Hoofdstuk 5 beschrijft onderzoek naar de effecten van het geneesmiddel ketoconazol op de groei, cel cyclus, apoptose en afgifte van ACTH of serotonine en chromogranine in respectievelijk een ACTH-producerende long NET cellijn en een niet-ACTH producerende pancreas NET cellijn. Ketoconazol heeft een voornamelijk pro-apoptotisch of cytotoxisch effect, afhankelijk van het type cellijn. Bovendien zorgt behandeling met ketoconazol voor veranderingen in celcyclus, namelijk een toename in G0/G1 fase in beide cellijnen en een afname in G2/M fase in de pancreas NET cellijn. Bovendien tonen de resultaten in hoofdstuk 5 een niet eerder gerapporteerd remmend effect aan van het SSA pasireotide op serotonine afgifte van de pancreas NET cellijn. Dit effect wordt waarschijnlijk gemedieerd via de hoge SST_5 expressie in deze cellijn.

Het onderzoek in **hoofdstuk 6** beschrijft een uitgebreide karakterisering van twee pancreas NET modellen in tweedimensionale (monolayer) en driedimensionale (sferoïden) kweeksystemen, in verschillende kweekmedium condities. Op basis van de resultaten van dit onderzoek wordt geconcludeerd dat een driedimensionaal (sferoïde) kweeksysteem een veelbelovend systeem is om groei en hormoon secretie van NET cellijnen te bestuderen, zelfs onder serum arme kweekcondities. In dit onderzoek wordt tevens aangetoond dat de expressie van SSTs en de dopamine D2 receptor (D2R) verandert tijdens de groei van cellen, zowel in twee- als driedimensionale kweken. De effecten van het somatostatine analoog octreotide, de dopamine agonist cabergoline en van twee nieuwe SSTs-D2R bindende geneesmiddelen (BIM-065 en BIM-23A760) werden onderzocht in de twee kweeksystemen. Cabergoline en de twee SSTs-D2R multi-receptor bindende geneesmiddelen, en niet octreotide, hadden een remmend effect op de afgifte van chromogranine en serotonine, maar niet op de celgroei. De bevindingen suggereren dat het effect van de twee SSTs-D2R bindende geneesmiddelen op de afgifte van hormonen in dit pancreas NET model verloopt via de D2R, en een mogelijke rol voor dopamine agonisten bij de behandeling van D2R-bevattende functionele NET, waaronder het carcinoid syndroom.

Hoofdstuk 7 beschrijft onderzoek naar de *in* vitro effecten van de nieuwe tryptofaan hydroxylase remmer telotristat op pancreas NET cellen. Telotristat remt krachtig en dosisafhankelijk de serotonine afgifte, bij klinisch relevante concentraties. De combinatie met pasireotide, maar niet met octreotide, had een aanvullend remmend effect op de serotonine afgifte. In driedimensionaal gekweekte pancreas NET cellen kon de serotonine afgifte volledig worden geremd door telotristat, maar was er geen effect op de celgroei. Deze resultaten tonen aan dat serotonine in dit pancreas NET model geen autocrien/paracrien effect heeft op de celgroei.

Het onderzoek in **hoofdstuk 8** toont dat de SSTs expressie in GEP-NET weefsel van patiënten met type 2 diabetes verlaagd is ten opzichte van GEP-NET weefsel van patiënten zonder diabetes. In type 2 diabetes patiënten die behandeld werden met metformine werd deze verlaging in SSTs expressie niet gevonden. *In vitro* experimenten toonden groei remmende effecten van biguanides en statines aan op NET cellijnen, met een toegenomen apoptose en afgenomen migratie van de cellen. Aangezien metformine en statines commercieel verkrijgbare en relatief goedkope geneesmiddelen zijn en er een langdurige ervaring is in het gebruik ervan, kunnen deze geneesmiddelen mogelijk van aanvullende waarde zijn bij de adjuvante behandeling van patiënten met NET.

In **hoofdstuk 9** worden de resultaten en conclusies van de studies uit dit proefschrift bediscussieerd in relatie tot huidige en mogelijk nieuwe toekomstige diagnostische en therapeutische mogelijkheden voor patiënten met een NET.

RESUMEN

Los tumores neuroendocrinos (NETs) son un grupo heterogéneo de neoplasias poco comunes, caracterizadas por un lento crecimiento y que se originan a partir de las células enterocromafines. Su incidencia anual viene aumentando progresivamente, aunque se desconoce si éste incremento es real, el reflejo de mayor uso de (mejores) técnicas diagnosticas o una combinación de ambas. A pesar de que frecuentemente los NETs tienen metástasis al diagnóstico, la supervivencia en estos pacientes ha aumentado, lo cual puede relacionarse con mejoras en las opciones diagnósticas y terapéuticas disponibles. Los NETs requieren métodos diagnósticos estandarizados para asegurar un diagnóstico precoz, así como para monitorizar la progresión de la enfermedad y orientar un tratamiento óptimo. Adicionalmente, el número y la calidad de las opciones terapéuticas se ha incrementado en los últimos años. En este sentido, esta tesis evalúa algunas nuevas opciones diagnósticas y terapéuticas para pacientes con NETs. El **capitulo 1** ofrece una visión general sobre los NETs, y se centra en la utilidad/limitaciones de los biomarcadores actualmente disponibles, así como en la descripción de algunos nuevos (posibles) marcadores en NETs. Además, resume opciones terapéuticas para tumores neuroendocrinos funcionantes y no funcionantes, tanto aquellas nuevas como las actualmente disponibles. Este capitulo representa la introducción al contenido fundamental de esta tesis: nuevos marcadores diagnósticos y opciones terapéuticas en NETs. El capitulo 1 termina con una visión general de los objetivos generales de esta tesis.

El **capitulo 2** evalúa detalladamente la expresión de algunos componentes de los sistemas somatostatina/cortistatina y ghrelina en una seria amplia, bien caracterizada, de carcinoides pulmonares típicos y atípicos. Hasta donde sabemos, este estudio representa la primera caracterización sistemática de los componentes de estos sistemas reguladores en muestras de carcinoides pulmonares (LCs), tejido adyacente no tumoral y pulmón sano. En este estudio, la expresión de somatostatina, de algunos de sus receptores (SSTs) y del receptor de ghrelina se incrementó gradualmente en el tejido no tumoral adyacente y en el tejido tumoral en comparación a pulmón normal. Además, la presencia de isoformas truncadas SST_5TMD_4, In1-ghrelin y el receptor de ghrelina GHSR1b fue reportada por primeras vez en LCs. Asimismo, una expresión aumentada de la enzima ghrelin-O-aciltransferasa (GOAT) se observó en tumores con necrosis, los cuales (a su vez) fueron aquellos con mayor tamaño, mayor capacidad de invasión y de producir metástasis a distancia. Estos datos sugieren que una desregulación del sistema ghrelina puede estar involucrada en el desarrollo/progresión de estos tumores.

El **capitulo 3** discute específicamente la expresión de componentes de los sistemas somatostatina/cortistatina en tumorres gastroenteropancreáticos (GEP-NETs). La sobre-expresión observada de SST_5 en el tejido tumoral comparado con el tejido adyacente no tumoral y el control sano, así como su relación clínica con la invasión vascular y nerviosa del tumor, sugieren un probable rol de SST_5 como diana terapéutica en GEP-NETs agresivos, además,

enfatiza la importancia de evaluar el perfil de SSTs en GEP-NETs, el cual aportaría información adicional para la toma de decisiones terapéuticas, incluida la utilización de análogos de somatostatina (SSAs) de segunda generación.

El hallazgo más novedoso y relevante del **capitulo 4** es la marcada sobre-expresión de la enzima GOAT en muestras de GEP-NETs. Específicamente, cuando la expresión de esta enzima es prácticamente ausente en tejido normal, su expresión se observó en tejido adyacente no tumoral y se sobre-expresó en tejido tumoral, una sobre-expresión que fue confirmada utilizando inmunohistoquímica. Asimismo, esta enzima se asoció con tumores de mayor tamaño, especialmente en NETs gastrointestinales. Estos hallazgos, combinados con publicaciones previas, sugieren el posible papel de la enzima GOAT como un biomarcador en NETs.

El **capitulo 5** describe los efectos de ketoconazol en la proliferación, ciclo celular, apoptosis, secreción de adrenocorticotropina (ACTH) o serotonina y cromogranina en una línea celular de NET bronquial productora de ACTH y una línea celular de NET pancreático no productora de ACTH. Específicamente, ketoconazol mostró características predominantemente pro-apoptóticas o citotóxicas dependiendo de la línea celular; estos efectos se mantuvieron a pesar de la combinación con SSAs. Además, ketoconazol fue capaz de inducir cambios en el ciclo celular, específicamente el aumento de las fases G0/G1 en ambas líneas celulares y una disminución de las fases G2/M en la línea celular pancreática. Incluso se observó un efecto inhibitorio en la secreción de serotonina del análogo de somatostatina pasireotide en la línea celular pancreática, el cual no había sido previamente descrito y que probablemente se debe a la expresión de SST_5 en esta línea celular.

En el **capitulo 6** se caracteriza de forma detallada un modelo de líneas celulares pancreáticas utilizando sistemas de cultivo en dos- (2D; monolayer) y tres- dimensiones (3D; esferoides) en diferentes condiciones de nutrientes. Al analizar los resultados, se observa que los cultivos en 3D con esferoides representan un método valioso para evaluar la secreción y proliferación celular en líneas celulares de NETs, incluso en condiciones con deprivación de suero. Se observó una expresión dinámica de los SSTs y receptor de dopamina (D2R) en los sistemas de cultivo en 2D y 3D, lo cual no se había descrito previamente. En este capítulo, los efectos del análogo de somatostatina octreotide y el agonista de dopamina cabergolina, así como de los nuevos fármacos quimera SSTs-D2R (BIM-065, BIM-23A760) se evaluaron en ambos modelos de cultivo. El tratamiento con cabergolina y ambas quimeras inhibió la secreción de cromogranina A y serotonina, pero no la proliferación celular. Esto sugiere que el efecto de las quimeras en la secreción parece estar mediado por el D2R, sugiriendo un posible papel de los agonistas de cabergolina para el tratamiento de NETs funcionantes, incluido el síndrome carcinoide siempre y cuando expresen D2R.

El **capitulo 7** muestra los efectos *in vitro* de telotristat etiprate, un nuevo inhibidor de la enzima hidroxilasa de triptófano en líneas celulares pancreáticas. Este fármaco mostró un potente efecto en la inhibición de la secreción de serotonina, el cual fue dependiente de

dosis en un modelo de células pancreáticas de NETs. Su combinación con pasireotide, pero no con octreotide, tuvo un efecto aditivo en el control de la secreción de serotonina. En el modelo 3D de NET pancreático, la secreción de serotonina fue totalmente inhibida por telotristat, sin embargo, este bloqueo no produjo cambios en el crecimiento celular. Estos datos sugieren que la serotonina no ejerce un efecto autocrino/paracrino en la proliferación celular en este modelo 3D de NET pancreático.

En el **capitulo 8** se observó una expresión disminuida de SSTs en pacientes con diabetes tipo 2 y GEP-NETs comparado con pacientes con GEP-NETs pero sin diabetes. Esta expresión disminuida no se observó cuando los pacientes diabéticos fueron tratados con metformina. Los estudios *in vitro* demostraron el efecto anti-proliferativo de las biguanidas y estatinas en líneas celulares de NETs, con el consiguiebnte incremento de la apoptosis y disminución de la migración celular. Puesto que la metformina y las estatinas son fármacos comercialmente disponibles, económicos y con una amplia experiencia en su uso, nuestros resultados sugieren que estos fármacos podrían representar una opción terapéutica factible como terapia adyuvante en NETs.

Finalmente, en el **capitulo 9** se discuten los resultados y conclusiones de los estudios presentados en esta tesis, especialmente en relación a las modalidades diagnósticas y opciones terapéuticas, tanto actuales como futuras, para los pacientes con NETs.

Appendices

PhD Portfolio

List of publications

About the author

Acknowledgements

PhD PORTFOLIO

Erasmus MC Department	Internal Medicine- Section of Endocrinology
Reina Sofia UH Department	Endocrinology and Nutrition Unit
Research School	Molmed
	Maimonides Institute of Biomedical research
Research Period	2014-2018
Promotors	Leo J. Hofland/Justo P. Castaño
Co-promotors	Richard A. Feelders/María Angeles Gálvez

Erasmus General Academic Courses	**Year**	**Workload (ECTS)**
The Introduction in GraphPad Prism Version 6	2018	0.3
Workshop on Microsoft Access 2010: Advanced	2018	0.3
Workshop on Microsoft Access 2010: Basic	2018	0.3
Course Bayesians statistics and JASP	2018	0.3
Biomedical English Writing Course for MSc and PhD-students	2018	2.0
Microscopic Image Analysis: From Theory to Practice	2018	0.8
Workshop Writing Successful Grant Proposals	2017	0.5
Research Integrity	2017	0.3

Clinical Courses and meetings	**Year**	**Workload (ECTS)**
Post-graduate course 15th Annual Conference for diagnosis and treatment of neuroendocrine tumor disease. *European Neuroendocrine Tumor Society*	2018	5
Course in systematic reviews and metaanalysis. *Reina Sofia University Hospital. Maimonides Institute of Biomedical Research*	2017	4.5
Problems and solutions for treating osteoporosis. *Spanish Society of the Bone and Mineral Research*	2017	
Clinical scenarios for type 2 diabetes. *Córdoba Medical College*	2017	0.4
Pre-conference course 14th Annual Conference for diagnosis and treatment of neuroendocrine tumor disease. *European Neuroendocrine Tumor Society*	2017	3
Course in Clinical Nutrition. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2017	0.6
I meeting for heart and diabetes. *Andalusian Society of Endocrinology, Diabetes and Nutrition*		
IX meeting in neuroendocrinology. *Spanish Society of Endocrinology and Nutrition*	2017	
Update course in diabetes. *Andalusian Health Board*	2016	2
Pre-conference course in neuroendocrinology. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2016	
Mellanby centre Training Course in osteoporosis. *Spanish Society of the Bone and Mineral Research*	2016	0.8

Actuality in Osteoporosis Spanish Society of the Bone and Mineral Research	2016	0.6
XXI update course in neuroendocrinology and nutrition. *Spanish Society of Endocrinology and Nutrition*	2016	2
XVII advanced course in diabetes. *Spanish Society of Endocrinology and Nutrition*	2016	
Appropriate use of antimicrobials. *Andalusian Health Board*	2015	1
Update in enteral nutrition. *Reina Sofia University Hospital*	2015	
Translational research in rare diseases. *Maimonides Institute of Biomedical Research*	2015	0.8
Pre-conference course in thyroid cancer. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2015	
XXI update in clinical nutrition. *Spanish Society of Endocrinology and Nutrition*	2015	
Diabetes Live. Attention to the patient with diabetes		0.5
Cardiovascu.ar health in diabetic patients with enteral nutrition	2015	0.8
Course of thyroid ultrasound for endocrinologists. *Reina Sofia University Hospital*	2015	0.6
Course of diagnostic and therapeutic neck ultrasound. *Reina Sofia University Hospital. Andalusian Health Board*	2015	1
Diabetes live! Patients with Diabetes. *Andalusian Health Board*	2015	6.88
III course in scientific divulgation. University of Córdoba	2015	1
Pre-conference course in thyroid ultrasound. *Andalusian Society of Endocrinology, Diabetes and Nutrition*		
Nutritional support in elderly. *Spanish Society of Enteral and Parenteral Nutrition*	2014	4.2
III course in neuroendocrinology. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2014	1.82
Management of the diabetic patient in the hospital and after the delivery. *Andalusian Health Board*	2014	0.57
8th update course in Diabetes. *Reina Sofia University Hospital*	2014	2
Update in Osteoporosis. *Reina Sofia University Hospital*	2014	0.4

Conferences oral/poster presentations	**Year**
Type 2 diabetes in neuroendocrine tumors: are biguanides and statins part of the solution? ***18th Congress of the European Neuroendocrine Association.***	2018
Hyperlipidemia during gestational diabetes: maternal and offspring complications. *20th European Congress of Endocrinology.*	2018
Utility of 3D cell culture systems with spheroids in neuroendocrine tumors. *20th European Congress of Endocrinology.*	2018
Effects of multi-receptor targeting drugs in neuroendocrine tumors using 3D cell culture. *15th congress of the European Neuroendocrine Tumor Society*	2018
Potential effects of ketoconazole on ACTH-producing and non- ACTH-producing neuroendocrine tumors. *15th congress of the European Neuroendocrine Tumor Society.*	2018

Ghrelin O-acyltransferase (GOAT) enzyme and ghrelin receptor GHSR1a as putative prognosis markers and therapeutic targets in gastroenteropancreatic neuroendocrine tumors. *15th congress of the European Neuroendocrine Tumor Society.*	2018
Effects of ketoconazole on ACTH-producing and non ACTH-producing neuroendocrine tumor cells. *19th European Congress of Endocrinology*.	2017
Potential anti-tumor activity of biguanides and statins in neuroendocrine tumor cells. *14th European Neuroendocrine Tumor Society Conference.*	2017
Planning for pregnancy in type 1 diabetes *XXVIII conference of the Spanish Diabetes Society*	2017
Muscular area by computed tomography in liver transplant patients *IX National Conference for the Clinical Assistance of the Chronic Patient of the Spanish Society of Internal Medicine*	2017
Role of the computed tomography in the evaluation of the body composition in liver transplant patients *XIX conference of the Spanish Society of Musculoskeletal Radiology*	2017
Components of Splicing Machinery are Drastically Dysregulated on Neuroendocrine Tumors and Associated with Malignancy. *17th Congress of the European Neuroendocrine Association.*	2016
Early nutrition support therapy in patients with head-neck tumors. *38th Conference of the European Society of Parenteral and Enteral Nutrition*	2016
Radiation and chemotherapy side effects in patients with head-neck tumors receiving early nutrition support therapy. *38th Conference of the European Society of Parenteral and Enteral Nutrition*	2016
The role of body composition evaluation by computerized tomography in pre-liver trasplant patients. *38th Conference of the European Society of Parenteral and Enteral Nutrition*	2016
Adrenal incidentalomas: functionality study. *18th European Congress of Endocrinology*	2016
Presence and clinical-histological correlates of ghrelin and somatostatin systems components in gastroenteropancreatic neuroendocrine tumors and lung carcinoids. *18th European Congress of Endocrinology*	2016
Splicing Dysregulation Impacts on Neuroendocrine Tumors: Evidence from Altered Spliceosoma Components and Somatostatin and Ghrelin Systems. *13rd European Neuroendocrine Tumor Society Conference.*	2016
Thyroid nodule: from the finding to the diagnosis of thyroid cancer *58th conference of the Spanish Society of Endocrinology*	2016
Effects of ketoconazole on ACTH-producing and non ACTH-producing neuroendocrine tumor cells. *58th conference of the Spanish Society of Endocrinology*	2016
Percutaneous endoscopic gastrectomy: Third level Hospital Experience *58th conference of the Spanish Society of Endocrinology*	2016
Clinical evaluation of gestational diabetes *XXVII conference of the Spanish Diabetes Society*	2016

Delivery outcome in patients with type 1 diabetes *XXVII conference of the Spanish Diabetes Society*	2016
Nutritional Evaluation In Pre-Liver Transplant Patients. *37th Conference of the European Society for parenteral and Enteral Nutrition*	2015
Nutritional Evaluation In Pre-Lung Transplant Patients. *37th Conference of the European Society for parenteral and Enteral Nutrition*.	2015
Expression of ghrelin and somatostatin systems components in pancreatic neuroendocrine tumours and their relationship with clinical-histological characteristics. *17th European Congress of Endocrinology*.	2015
Bariatric surgery and reduction in cardiovascular risk. *36th Congress of the European Society of Parenteral and Enteral Nutrition*	2014
Clinical, histological and molecular characteristics of gastroenteropancreatic neuroendocrine tumors *57th conference of the Spanish Society of Endocrinology*	2015
Combination of invasive techniques and intraoperatory ultrasound for the identification of insulinomas *57th conference of the Spanish Society of Endocrinology*	2015
Differences in the diagnostic of gestational diabetes according to the oral glucose tolerance test criteria *57th conference of the Spanish Society of Endocrinology*	2015
Differences in the molecular profile of pituitary adenomas after the second surgery *57th conference of the Spanish Society of Endocrinology*	2015
In differentiated thyroid cancer, is useful a second stimulatory test with rhTSH in patients with negative post-surgery control? *57th conference of the Spanish Society of Endocrinology*	2015
Bone and mineral metabolism evolution after bariatric surgery *XXVI conference of the Spanish Diabetes Society*	2015
Determining factors in the metabolic control of type 1 diabetes *XXVI conference of the Spanish Diabetes Society*	
Efficacy of denosumab in menopausal osteoporotic patients who require to suspend bisphosphonates *XIX conference of the Spanish Society of the Bone and Mineral Research*	2014
Glutamine and total parenteral nutrition in bone narrow transplant patients. *36th Congress of the European Society of Parenteral and Enteral Nutrition*	2014
Lateral amyotrophic sclerosis: enteral nutrition using percutaneous endoscopic gastrectomy *56th conference of the Spanish Society of Endocrinology*	2014
Subclinical hypothyroidism associated with thyroid autoimmunity *56th conference of the Spanish Society of Endocrinology*	2014

Awards and prizes/Travel grants	Year
European Neuroendocrine Association Travel Grant. *European Neuroendocrine Association*	2018
European Conference Travel Grant. *Spanish Society of Endocrinology and Nutrition*	2018
Basic Science Travel Grant. *European Society of Endocrinology*	2018
Double doctorate grant. *University of Córdoba*	2018
Excellence Prize for Endocrinology Residents. *Spanish Society of Endocrinology and Nutrition*	2017
Rio Hortega Research Grant. *Institute of Health Carlos III*	2017
Best Oral Presentation. *Andalusian Society of Clinical Nutrition and Dietetics*	2016
Basic Science Travel Grant. *European Society of Endocrinology*	2016
Colaboration for short internships. *Spanish Society of Diabetes*	2016
European Conference Travel Grant. *Spanish Society of Endocrinology and Nutrition*	2015
Best oral presentation Basic Endocrinology. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2015

LIST OF PUBLICATIONS

Publications in peer reviewed journals

1. Aura D Herrera-Martínez, Leo J. Hofland, Wouter W. de Herder, María A. Gálvez Moreno, Justo P. Castaño and Richard A. Feelders. Neuroendocrine tumors: diagnostic, predictive and prognostic markers. Endocrine related Cancer. Submitted
2. Aura D Herrera-Martínez, Johannes Hofland, Leo J. Hofland, Tessa Brabander, Ferry Eskens, María A. Gálvez Moreno, Justo P. Castaño, Wouter W. de Herder, Richard A. Feelders. Medical Treatment for neuroendocrine tumors: current options and future perspectives. Drugs. Submitted
3. Aura D Herrera-Martínez, Richard A. Feelders, Wouter W. de Herder, Justo P. Castaño, María Ángeles Gálvez Moreno, Fadime Dogan, Rosanna van Dungen, Peter van Koetsveld, Leo J. Hofland. Effects of ketoconazole on ACTH-producing and non-ACTH-producing neuroendocrine tumor cells. Submitted
4. Aura D Herrera-Martínez, Rosanna van den Dungen, Fadime Dogan-Oruc, Peter M. van Koetsveld, Michael D Culler, Wouter W. de Herder, Raúl M. Luque , Richard A. Feelders, Leo J. Hofland. Effects of novel somatostatin-dopamine chimeric drugs in 3D spheroid cell culture models of neuroendocrine tumors. Submitted
5. Aura D Herrera-Martínez, Richard A. Feelders, Rosanna van den Dungen, Fadime Dogan-Oruc, Peter M. van Koetsveld, Justo P. Castaño, Wouter W. de Herder, Leo J. Hofland. Efficacy of the tryptophan hydroxylase inhibitor telotristat on growth and serotonin secretion in 2D and 3D cultured neuroendocrine tumor cells. Submitted
6. Johannes Hofland, Aura D. Herrera-Martínez, Wouter T. Zandee, Wouter W. de Herder. Management of carcinoid syndrome: a systematic review and meta-analysis. Submitted
7. Aura D. Herrera-Martínez, Sergio Pedraza-Arevalo, Fernando L-López, Manuel D. Gahete, María A. Gálvez-Moreno, Justo P. Castaño, Raúl M. Luque. Type 2 diabetes in neuroendocrine tumors: are biguanides and statins part of the solution?. J Clin Endocrinol Metab. 2018 Sep 27. doi: 10.1210/jc.2018-01455.
8. Aura D. Herrera-Martínez, Manuel D. Gahete, Rafael Sánchez-Sánchez, Emilia Alors-Perez, Sergio Pedraza-Arevalo, Raquel Serrano-Blanch, Antonio J. Martínez-Fuentes, Maria A. Gálvez-Moreno, Justo P. Castaño, Raúl M. Luque. Ghrelin O-acyltransferase (GOAT) enzyme as a novel potential biomarker in gastroenteropancreatic neuroendocrine tumors. Clinical and Translational Gastroenterology. Clin Transl Gastroenterol. 2018 Oct 8;9(10):196.
9. Aura D. Herrera-Martínez., Rodrigo Bahamondes, Rafael Palomares Ortega, Paloma Moreno Moreno, M. Angeles Gálvez. Hyperlipidemia during gestational diabetes: maternal and offspring complications. Nutrición Hospitalaria. In Press

10. María Rosa Alhambra Expósito; Aura D Herrera-Martínez; Gregorio Manzano García; María Espinosa Calvo; Carmen María Bueno Serrano; María Ángeles Gálvez Moreno. Early nutrition support in head neck cancer patients. Nutrición Hospitalaria. In Press
11. Aura D. Herrera-Martínez, Manuel D. Gahete, Se, io Pedraza-Arevalo, Rafael Sánchez-Sánchez, Rosa Ortega-Salas, Raquel Serrano-Blanch, María A. Gálvez-Moreno, Raúl M. Luque, Justo P. Castaño. Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors. Endocrine. 2018 Feb;59(2):426-437. doi: 10.1007/s12020-017-1482-3.
12. Aura D Herrera-Martínez; Manuel D. Gahete; Rafael Sánchez-Sánchez; Rosa Ortega Salas; Raquel Serrano-Blanch; Ángel Salvatierra; Leo J. Hofland; Raúl M. Luque; María A. Gálvez-Moreno; Justo P. Castaño. The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features. Lung Cancer. 2017 Jul;109:128-136. ISSN 0169-5002
13. Aura D. Herrera-Martínez; Patricia Enes; María Martín-Frias; Belén Roldán; Rosa Yelmo; Raquel Barrio. La respuesta monofásica a la sobrecarga oral de glucosa como factor predictivo del riesgo de diabetes tipo 2 en pacientes pediátricos con obesidad. Anales de Pediatría, 2017, ISSN 1695-4033, http://dx.doi.org/10.1016/j.anpedi.2017.01.009.
14. Aura D. Herrera-Martínez; José Carlos Padillo Cuenca; Rodrigo Bahamondes Opazo; Ana Barrera Martín; Angel Rebollo Roman; Carlos Díaz Iglesias; María A. Gálvez Moreno. ACTH Producing Pancreatic Neuroendocrine Tumors in Multiple Endocrine Neoplasia Type 1. Journal of the Pancreas. ISSN 1590-8577
15. Paloma Moreno Moreno; María Rosa Alhambra Expósito; Aura Dulcinea Herrera Martínez; Rafel Palomares Ortega; Luis Zurera Tendero; Juan José Espejo Herrero; María Angeles Gálvez Moreno. Arterial Calcium Stimulation with Hepatic Venous Sampling in the Localization Diagnosis of Endogenous Hyperinsulinism. International journal of endocrinology. 2016, pp. 4581094. ISSN 1687-8337
16. Aura D Herrera Martínez; Rodrigo Bahamondes Opazo; Rafael Palomares Ortega; Concepción Muñoz Jiménez; Maria A. Gálvez Moreno; José M. Quesada Gómez. Primary hyperparathyroidism in pregnancy: a two-case report and literature review. Case reports in obstetrics and gynecology. 2015, pp. 171828. 2015. ISSN 2090-6684
17. Aura D Herrera Martínez; José C Padillo Cuenca; Alfonso Calañas Continente; Rodrigo Bahamondes Opazo; Concepción Muñoz Jiménez; María A Gálvez Moreno. Invasive techniques and intraoperative echography in the localization of insulinomas; a case report. Nutrición Hospitalaria. 32 - 1, pp.426 - 435. 2015. ISSN 1699-5198
18. Aura Herrera Martínez; Montserrat Viñals Torràs; Ma Concepción Muñoz Jiménez; Antonio Pablo Arenas de Larriva; Ma José Molina Puerta; Gregorio Manzano García; Ma Ángeles Gálvez Moreno; Alfonso Calañas Continente. Metabolic encephalopathy secondary to vitamin D intoxication. Nutrición Hospitalaria. 31 - 3, pp. 1449- 1500. 2015. ISSN 1699-5198

19. Aura Dulcinea Herrera Martínez; María R Alhambra Expósito; Gregorio Manzano García; María J Molina Puertas; Alfonso Calañas Continente; Rodrigo Bahamondez Opazo; Concepción Muñoz Jiménez; Rafael Rojas Contreras; María A Gálvez Moreno. Use of glutamine in total parenteral nutrition of bone marrow transplant patients. Nutrición hospitalaria. 31 - 4, pp. 1620 - 1624. 2015. ISSN 1699-5198
20. Inmaculada Prior Sánchez; Aura Dulcinea Herrera Martínez; Carmen Tenorio Jiménez; María José Molina Puerta; Alfonso Jesús Calañas Continente; Gregorio Manzano García; María Ángeles Gálvez Moreno. Percutaneous endoscopic gastrostomy in a myotrophic lateral sclerosis. Experience in a district general hospital.Nutrición Hospitalaria. 30 - 6, pp. 1289 - 1383. 2014. ISSN 1699-5198
21. Aura D Herrera Martínez; Pedro Estrada Corona. Spondylo-epiphyseal dysplasia associated with craniosynostosis, cleft palate and mental retardation. A case report. Investigación clínica. 51 - 4, pp. 553 – 613. 12/2010. ISSN 0535-5133.
22. Aura D Herrera Martínez; Alfonso J Rodriguez Morales. Trends in the seroprevalence of antibodies against Trypanosoma cruzi among blood donors in a Western Hospital of Venezuela, 2004-2008.Acta tropica. 116 - 1, pp.115 - 122. 10/2010. ISSN 1873-6254
23. Aura D Herrera Martinez; Alfonso J Rodríguez Morales. Potential influence of climate variability on dengue incidence registered in a western pediatric Hospital of Venezuela. Tropical biomedicine. 27 - 2, pp. 280 - 286. 08/2010. ISSN 0127-5720
24. Alfonso J Rodriguez Morales; Aura D Herrera Martínez; Yiraldine Herrera Martínez. Imported cases of malaria admitted to a hospital in Western Venezuela, 1998-2009. Travel medicine and infectious disease. 8 - 4, pp. 269 -340. 07/2010. ISSN 1873-0442.
25. Aura D Herrera Martínez; Yiraldine Herrera Martínez; Antonio Maria Pineda. Neurological opportunistic diseases in HIV infected patients in a western hospital of Venezuela, 2007 -2009.Brazilian journal of infectious diseases. 14 - 6, pp. 643 - 647. 2010. ISSN 1678-4391

ABOUT THE AUTHOR

Aura Dulcinea Herrera Martínez was born in Barinas, Venezuela. After finishing high school, she started her medical training at the Centrooccidental Lisandro Alvarado University in Barquisimeto, Venezuela, where she graduated Magna cum laude (2011). Subsequently she worked as a medical doctor in the Rafael Rangel Hospital in Yaritagua, Venezuela. Concurrently she studied education, defended a thesis on Learning Styles in Medical Universities (awarded Honorable Mention) and received the title of Magister Scientiarum in Higher Education (2013). In 2013 she started specializing in Endocrinology and Nutrition at the Reina Sofia University Hospital in Córdoba, Spain, under the supervision of dr. Rafael Palomares Ortega and dr. María Angeles Gálvez Moreno. Simultaneously she started research in neuroendocrine tumors as part of a Master Program in Translational Research under supervision of Prof. dr. Justo Castaño and Prof. dr. Raúl Luque. In 2015 she started her PhD training in the University of Córdoba, under supervision of Prof. dr. Justo Castaño, Prof. dr. Raúl Luque and dr. María Ángeles Gálvez Moreno.

In 2016 Aura performed a clinical/research rotation in the Erasmus Medical Center in Rotterdam, the Netherlands, under the supervision of Prof. dr. Leo J. Hofland, Prof. dr. Wouter de Herder and dr. Richard Feelders, and subsequently started a combined PhD training in both the University of Córdoba and the Erasmus University Rotterdam.

She finished her clinical training in Endocrinology and Nutrition in 2017, and received the 2017 Resident Award of the Spanish Society of Endocrinology and Nutrition. Since then she worked as a researcher at the department of Internal Medicine, section Endocrinology of the Erasmus Medical Center under the supervision of Prof. dr. Leo J. Hofland and dr. Richard Feelders. Currently she works as a consultant/researcher in Endocrinology at the Reina Sofia University Hospital and the Maimonides Institute of Biomedical Research of Córdoba.

ACKNOWLEDGMENT / DANKWOORD / AGRADECIMIENTO

This thesis would have not been possible without the help of numerous persons from different institutions and countries, and without the generous participation of several patients. I would like to thank all people, who directly and indirectly, supported me and this project during these years.

My deepest appreciation goes out to my supervisors Prof. dr. L.J. Hofland and Prof. dr. J.P. Castaño, to my co-supervisors dr. R.A. Feelders and M.A. Gálvez, as well as to their valuable research teams.

Beste *Leo*, wat was ik blij met mijn eerste, kleine stage in het Neuroendo lab Ik had nooit gedacht dat dit het begin van een dubbele doctoraat was. Dank voor de persoonlijke samenwerking, je kritische opmerkingen, je uitleg en alle Skype meetings. In het speciaal bedankt voor al het vertrouwen. Het was een enorm goed besluit om bij jou onderzoek te gaan doen.

Gracias *Justo* por la oportunidad de trabajar en vuestro grupo, por guiar esta tesis a pesar de los múltiples inconvenientes, pero, sobre todo, gracias por la paciencia y el optimismo ante las dificultades. Con tus consejos me has motivado a esforzarme al máximo y a dar lo mejor de mí, gracias.

Beste *Richard*, dank voor het prachtige maanden dat ik in de kliniek onder jouw supervisie heb mogen werken. Bedankt voor je positieve commentaren en enthousiaste reacties over de mooie resultaten. Het was een enorm plezier om met jou samen te werken.

Querida *María Angeles*, con tu iniciativa empezó mi trabajo en esta línea. Eres un gran ejemplo, como especialista y como persona, agradezco mucho tus enseñanzas, y en lo personal, la consideración, preocupación y el apoyo que me has dado.

Overige leden van de promotiecommissie: Prof. dr. *W.W. de Herder*, Prof. dr. *E-J.M. Speel*, Prof. dr. *C.H.J van Eijck,* hartelijk dank voor het kritisch doorlezen van dit manuscript en het plaatsnemen in de oppositie bij de verdediging van mijn proefschrift. Beste *Wouter*, ik wil je voor je uitleg, advies en vertrouwen bedanken. Ik heb veel van jou geleerd: niet alleen over endocrinologie, maar ook over Nederlandse geschiedenis, geografie en cultuur. Dank je voor je tijd. Je bent een ongelooflijk voorbeeld als professor en endocrinoloog.

To all my co-authors: thanks for your valuable comments and constructive feedback.

Raúl, has sido un director de tesis para mi, mil gracias por tu tiempo y dedicación, he sido muy afortunada por contar con tu orientación y apoyo tanto a nivel personal como profesional. *Manuel*, tus consejos, correcciones y enseñanzas son poco en comparación a tu disposición a escucharme y tenderme tu mano amiga; siempre estaré agradecida.

Beste *Hans*, ik wil je danken voor de tijd van warmte, steun en plezier. Ik was erg gelukkig om op je vriendschap te vertrouwen.

A mis compañeros de laboratorio (GC-08 y GC-24), especialmente a *Sergio* y *Fernando*, por su ayuda en el laboratorio cuando estuve en el extranjero, y a *Mari Carmen*, quien desde mi primer día en Rabanales me adoptó como su alumna.
Ik wil graag ook mijn master en PhD collega's van het Neuroendo lab bedanken voor de leuke momenten en tijd van samenwerken. *Anela*, *Peter* en *Fadime*, dank voor jullie uitleg, hulp en aardige gesprekken. Beste *Rosanna*, dank voor je betrokkenheid, organisatie en toewijding met mijn projecten.
Dear *Maarten*, thanks for your advice, support and enthusiasm. You taught me that obstacles are only opportunities for bigger projects; this thesis is one example. Thanks a lot!

A mi amada familia, por el apoyo incondicional a pesar de la distancia. Gracias *Abuela* por tus oraciones. Agradezco a mis tías *Valle* y *Mena* por ser fuente de aliento y ánimo. *Yiri,* gracias por estar siempre que te he necesitado, y por hacer en esos momentos el papel de hermana mayor. Gracias *Victor,* por contagiarnos con tu risa y alegría aún en la tristeza. Finalmente, y de forma muy especial, quiero agradecer a mis padres, sin quienes nada habría sido posible. *Papá*, porque eres y siempre serás mi referente, el mejor ejemplo que alguien pueda desear. *Mamá*, mi gran amiga e inspiración, porque eres el principal motivo para seguir adelante.